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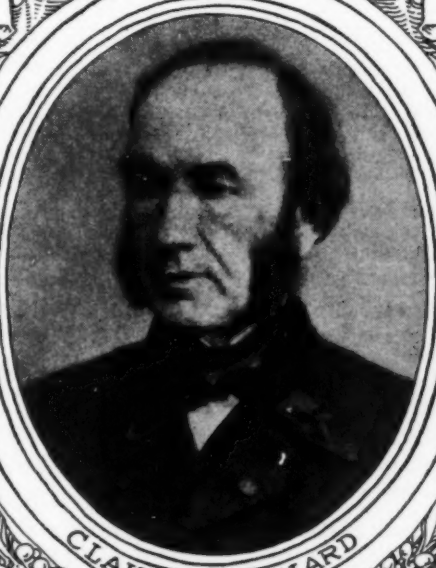

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HISTOLOGICAL STUDIES OF THE TISSUES OF RATS FED A DIET EXTREMELY LOW IN ZINC[1]

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TWO PLATES (ELEVEN FIGURES)

(Received for publication March 10, 1941)

Investigation at the University of Wisconsin (Todd, Elvehjem and Hart, '34; Stirn, Elvehjem and Hart, '35; Hove, Elvehjem and Hart, '37, '38) and recent studies in our laboratory (Day and McCollum, '40) have conclusively demonstrated the indispensability of zinc in the nutrition of the rat. The only pathological change that has been reported is a loss of hair about the neck and shoulders extending, sometimes, to involve the entire ventral surface of the body (Todd, Elvehjem and Hart, '34).

The present report deals with a detailed microscopic study of the tissues of rats which were the subject of a preliminary report (Day and McCollum, '40) on the preparation of a diet very low in zinc content, and observations on the growth, appearance, and phosphatase activity of blood, bone and kidney, as well as the carbonic anhydrase activity of blood.

MATERIALS AND METHODS

Histological observations were made on fourteen rats from three litters (table 1). The mothers of two litters were

[1] Aided by a grant from the Rockefeller Foundation to Dr. E. V. McCollum.

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restricted to the zinc-deficient diet when the young were 12 days old. The third litter was taken from the stock colony when the young were about 25 days old. The rats were kept on monel metal screens in pyrex glass jars. Pyrex glass rods, supported by wooden frames, were used to cover the jars. Precautions were taken to keep dust contamination at a minimum.

The preparation of the diet has been given elsewhere (Day and McCollum, '40). Seven rats were given the deficient diet and supplements. The total intake of zinc was not more than 2 to 4 μg. of zinc per rat daily. Seven littermate controls received the same diet and supplements plus 0.15 mg. Zn (as $ZnSo_4.7\,H_2O$) in 0.2 ml. of water per rat daily. The diet was given ad libitum. The daily supplement was composed of liver concentrate (equivalent to 6.4 gm. liver), 40 μg. of thiamine and 1.0 mg. of choline. Water was redistilled in pyrex glass.

The rats were killed with chloroform and immediately autopsied at the times shown by table 1. The tissues were fixed in Zenker-Formol solution, imbedded in paraffin, and stained with haematoxylin and eosin. Other special stains were used as indicated.

GROSS EFFECTS OF THE DEFICIENCY

Table 1 indicates the survival period of each animal. Three zinc-deficient rats died; two of litter no. 2 dying after 33 and 34 days respectively, and one of litter 3 dying after 61 days on the diet. Control animals were killed and autopsied at those times, as indicated in the table.

As shown in table 1, growth became greatly retarded in the Zn-deficient rats after the first 2 or 3 weeks. After 3 to 5 weeks, depending somewhat on the degree of deficiency as indicated by growth, the hair became sparser in all of the Zn-deficient rats, but at this time there were no completely denuded areas. Practically all of the subcutaneous fat disappeared early. After approximately 70 days Zn-deficient rats nos. 2 and 6 had developed definite areas of

alopecia. In no. 2, two denuded areas occurred in the mid-dorsal region, one measuring about 3.5×2.0 cm. and the other about 2.5×1.0 cm. In no. 6, the denuded area was over the right shoulder. The denuded spaces of nos. 2 and 6 were roughened and scaly. Apparently these areas were painful. A few days before the animals were killed they began to chew the afflicted parts, causing them to bleed

TABLE 1

Effect of dietary zinc deficiency on the growth and survival of rats

	RAT AND LITTER NUMBER	SEX	DAYS ON DIET[1]	WEIGHT IN GRAMS							
				Initial	2 wks.	4 wks.	6 wks.	8 wks.	9 wks.	10 wks.	11 wks.
Zinc deficient	1L3	M.	61 (d)	54	77	84	95	105	96		
	2L3	M.	74 (k)	40	55	51	67	75	76	77	78
	5L1	M.	50 (k)	37	51	65	70				
	6L1	Fe.	74 (k)	34	53	59	74	78	84	88	90
	7L1	M.	74 (k)	37	58	79	69	102	101	107	110
	10L2[2]	M.	33 (d)	23	41	40					
	11L2	Fe.	34 (d)	25	39	42					
	12L2	M.	34 (k)	28	42	48					
	Average weight			35	52	59	75	90	89	91	93
Zinc added	3L3	M.	74 (k)	38	64	100	154	180	189	200	207
	4L3	M.	61 (k)	46	81	125	179	223	234		
	8L1	Fe.	74 (k)	32	50	85	128	152	160	168	180
	9L1	M.	50 (k)	35	61	99	146				
	13L2	Fe.	74 (k)	24	55	77	98	121	131	140	155
	14L2	M.	33 (k)	24	62	89					
	15L2	M.	74 (k)	28	69	102	162	196	217	226	231
	Average weight			32	63	97	145	174	186	184	193

[1] K—killed; D—died.

[2] Animal died during the night. Tissues were not used for histological studies.

slightly. Number 7 rat developed alopecia over the lower back but dermatitis did not occur. Thus all three of the Zn-deficient rats that lived more than 61 days developed macroscopic skin lesions. The tails appeared normal. Considerable amounts of hair were found in the stomachs of all the Zn-deficient animals.

About 18 days before the experiment was ended one control female, no. 13, was placed in the cage of a control male.

When the female was autopsied six fetuses were found, giving additional evidence that the experimental diet was adequate except for zinc.

MICROSCOPICAL FINDINGS

Alimentary system. Since the oesophagus showed the most pronounced change this will be described first. The buccal lesions were less marked. The normal lining of the oesophagus consists of a basal layer of cells in which occasional mitotic figures are seen. On this are several layers with larger clearer nuclei whose cell borders are indistinct. There is an abrupt transition to a keratinized layer on which are usually adherent bacteria (figs. 1 and 3). In contrast to this the Zn-deficient animals showed a different picture. The cells of the basal layer were more numerous and closely packed but there were no more mitotic figures than in the controls. In the overlying stratum the cells were also more numerous and instead of being two or three layers thick, they averaged six or eight cells thick. The nuclei were a little smaller and the nuclear membrane was wrinkled. No mitotic figures were found in these cells. On the inner edge of this layer the nuclei became smaller and stained deeper but did not disappear as occurs normally. Instead there was a stratum consisting of small dark nuclei four or five layers deep imbedded in a pink-staining, homogeneous material. There was slight keratinization of the inner margin. There were large numbers of gram positive cocci adherent to the edge (figs. 2 and 4). The epithelium of the posterior roof of the mouth showed the same change though not to so marked a degree; no change was found in the anterior portion. In occasional foci the tongue exhibited the alteration too. The marked change in the oesophagus began sharply at the base of the tongue and extended throughout the entire length to its entrance into the fore stomach, at which point it ceased. The two portions of the stomach were normal as were the small and large intestines. The salivary glands, liver, and pancreas showed no change. The latter organ was stained

to bring out the cytology of the islet cells but no differences were noted.

Circulatory system. Sections of the heart and blood vessels from the Zn-deficient rats did not differ from their controls.

Respiratory system. The mucosa of the nares of the deficient animals was entirely normal as were the glands beneath. The trachea was lined by ciliated epithelium; the bronchi and pulmonary parenchyma were entirely normal.

Genito-urinary system. Normal spermatogenesis was taking place in the one male animal autopsied at 50 days. However, in rats autopsied after 61 and 74 days on the diet, although sperm was being formed in some of the tubules, there were numerous atrophic ones lined only by spermatogonia. Some contained giant cells such as are seen in chronic inanition. All the tubules were smaller than those of the controls. The epithelium of the accessory glands was not so columnar and these structures were smaller in the deficient rats.

In the ovaries, corpora lutea were not found in the deficient rats. The follicles had partially developed, however. The epithelial cells lining the uterus were lower and non-vacuolated; there were only a few glands as compared with those of the controls. One normal (no. 13) showed decidual tissue. The vagina of the deficient rats showed keratinized epithelium with leucocytes.

The kidneys, their pelves, the ureters and bladder were normal in all of the deficient animals.

Nervous system. Sections of the brain showed no noteworthy differences between the deficient animals and their controls.

Myeloid and lymphoid systems. The marrow of the long bones of the deficient animals showed no changes from the normal. The lymphoid tissue in the deficient animals was less conspicuous in the Zn-low animals. All spleens and thymus glands were smaller in the deficient animals than in their controls.

Skeleton. Sections of the upper tibiae revealed differences between the two groups. The epiphyseal cartilage was a little

narrower in the deficient animals and the hypertrophic cells were not as numerous. The trabeculae at the cartilage-shaft junction were thinner and shorter in the Zn-low animals than in their controls. There seemed to be fewer osteoblasts in the former group.

Muscle. No alterations in the smooth or striated muscle of the deficient animals could be found.

Ductless glands. The thyroid glands of the deficient rats tended to have smaller follicles but no other changes could be detected. The parathyroid glands of the Zn-deficient rats were not different from the controls. The adrenals of the deficient and control animals were alike. The hypophyses of the deficient animals were definitely smaller than those of the controls. Sections were stained to bring out the chromophilic granules. The number of basophile cells was about the same in both groups. The eosinophilic cells showed no very great difference in the two groups either.

Skin. No changes occurred in the skin of the control animals. Definite lesions were found in five out of the seven Zn-deficient rats (figs. 6–11). The earliest change which was found at 33 days consisted of a hyper-keratinization. Coincident with this there was a thickening of the epidermis. Instead of having a normal thickness of three to four cell layers, the epithelium increased to eight to ten cells in thickness. The epithelium lining the hair follicles became keratinized as well. As the epidermis increased in thickness, there was no appreciable increase in mitotic figures in the basal layer; however, mitoses could be detected in the cells lying above. Another prominent feature was the presence of clear spaces in the cytoplasm of the basal cells as well as those above and the nuclei became more pyknotic. The cells, too, became spread apart by fluid since intercellular spaces were noted. At this stage there was a crust lying on the epidermis consisting of keratinized fibers, bacteria and leucocytes. In the corium there was little or no oedema; but there were in later stages leucocytes, both polymorphonuclear as well as monocytes. In the later stages, too, corresponding with the

areas from which the hair had been lost, changes appeared in the follicles and these disappeared completely leaving only a few mononuclear cells to mark where they had been. The sebaceous glands, however, remained and the individual cells were larger. In none of the animals was there a disappearance of these structures. As noted grossly, this change took place over the dorsum of the animals. Similar microscopic changes about the nose were found in four out of seven deficient animals. No lesions were found on the plantar surface nor were there any changes in the ears.

Eyes. Lesions of the cornea were noted in two of the seven deficient animals (nos. 5 and 6). In the tunica propria there was a dense cellular infiltration consisting of mononuclear and polymorphonuclear leucocytes, with the former predominating. They were found throughout but were more numerous just beneath the epithelium. Blood vessels were also present (fig. 5). Descemet's membrane was intact. There was vesiculation of some of the basal epithelial cells and in places the epithelial cells were spread apart. There was no increased keratinization of the cornea or conjunctivae. No changes were found in the lacrimal glands.

DISCUSSION

We have presented a detailed histological study of the tissues of rats restricted to a dietary intake of 2 to 4 μg. Zn per rat daily. Changes were found in the oesophagus in all the animals and to a lesser extent in the mouth. Lesions were noted in the skin in five of the seven deficient animals and changes in the eyes in two of the seven Zn-deficient rats.

In the oesophagus, there was a thickening of the epithelial cell layer together with the presence of an inner layer of incompletely keratinized cells. No evidence of an increased proliferation of cells could be found as evidenced by mitotic figures. The changes can be interpreted as due either to a retardation in the normal keratinization of the epithelium which then is not removed or to an increased proliferation of cells. The change is identical with the parakeratosis which

one encounters in human skin lesions such as psoriasis. These changes were seen to a slighter degree in the buccal cavity. The epithelium lining the fore stomach was not involved. Such an alteration of the oesophagus in rats is unique. It does not resemble the lesions of vitamin A deficiency (Wolbach and Howe, '25) which does not involve the oesophagus nor did the deficient animals show changes associated with this deficiency in the other tissues.

The alterations in the skin consisted of a hyperkeratinization, thickening of the epidermis, intra- and intercellular oedema and loss of hair follicles with preservation of the sebaceous glands. Secondary bacterial invasion took place later. It is to be emphasized that the plantae and ears showed nothing. Skin lesions were found in five of the seven Zn-deficient rats.

Todd, Elvehjem and Hart ('34) noted a loss of hair on the ventral surfaces of the rats in which was demonstrated the indispensability of the Zn in the diet. In contrast, the alopecia and changes in the skin were most marked over the dorsum of our animals.

Changes in the skin of rats have been noted in a wide variety of deficiency states (Sullivan and Nicholls, '40 a). Until recently, however, accurate histological studies using purified diets lacking one or more known components have not been undertaken. The changes that have been described in the Zn-deficient animals have no counterpart, to our knowledge, in any of the other nutritional dermatoses in the rat. Sullivan and Nicholls ('40 b) have described the lesions in vitamin B_6 deficiency which consist of hyperkeratosis, acanthosis and oedema of the corium with leucocytic infiltration over the paws, nose, chin, submental region and upper chest. The sebaceous glands and hair follicles remain intact. In riboflavin deficiency these authors ('41) found partial alopecia over the venter with inflammation of the eyelids and spectacle alopecia in some animals. Microscopically, there is a disintegration of the sebaceous glands and disappearance of the hair follicles with atrophy of the epidermis. The change

associated with vitamin H deficiency (Gyorgy, Sullivan and Karsner, '37), or egg-white injury, consists of acanthosis with inter- and intra-cellular oedema, hyperkeratosis and atrophy of the hair follicles and sebaceous glands. From the present state of our knowledge, it seems safe to conclude that Zn-deficiency produces a definite pathological picture in the skin of the rat.

As noted above, corneal lesions were found in two of the seven deficient animals. This change consisted of vascularization and leucocytic infiltration. The picture seems similar to that recently described by Bessey and Wolbach ('39) in uncomplicated riboflavin deficiency in rats. These authors noted extreme vascularization followed later by leucocytic infiltration. They were unable to produce the change by other means, such as fasting, old age, vitamin B_2 and B_6 deficiency and it disappeared when riboflavin was added to the diet.[3] The same vascular proliferation and cellular infiltration have been noted in vitamin A deficiency by Wolbach and Howe ('25). It must be pointed out that since the corneal epithelium showed no evidence of characteristic keratinization this deficiency may be dismissed. In our present state of information and since the change occurred in so few animals, we do not feel justified in drawing any conclusions as to the effect of Zn-deficiency on the cornea.

As noted above, changes were found in the gonads and accessory sexual organs. The atrophy of the testicular tubules and partial lack of spermatogenesis together with the failure of the ovarian follicles to mature were interpreted to be due to inanition and we did not feel they were produced directly by Zn-deficiency. The changes in the secondary sexual organs were those characteristic of retarded development and were doubtless also associated with partial inanition.

The skeleton showed a slowing of growth and the histological picture was a non-specific one such as is seen in athreptic

[3] In a recent personal communication Dr. Wolbach writes, "the one preparation of the cornea that you sent seems to be very much like the riboflavin-deficiency corneas".

animals. This was interesting since chemical analyses showed that the average concentration of Zn in the femurs of the deficient rats was only 94.7 μg. per gram of ash as compared with 236.6 μg. per gram of ash in the controls.

Since Zn has been shown to be an important constituent of insulin, the islets of Langerhans in the pancreas were studied with interest. No changes could be detected, however. It may be pointed out that Hove, Elvehjem and Hart ('38) found no disorder of carbohydrate metabolism in the animals they placed on a low Zn diet. These workers postulated that there was a decreased absorption of foodstuffs through the intestinal mucosa.

The activity of intestinal phosphatase (Hove, Elvehjem and Hart, '40 a), blood phosphatase (Day and McCollum, '40) and carbonic anhydrase (Hove, Elvehjem and Hart, '40 b) is decreased by a deficiency of zinc. Also, the absorption of protein is impaired by zinc deficiency (Hove, Elvehjem and Hart, '38). Since riboflavin is essential in the metabolism of amino acids, as a flavin-adenine-dinucleotide (Theorell, '40), it seems permissible, in view of the findings reported here, to hypothesize that a deficiency of zinc might affect the respiratory functions of the flavoproteins. In view of this the possibility of a relationship between zinc and the metabolism of riboflavin is being investigated by one of us (H. G. D.).

SUMMARY

In rats fed a diet adequate in all known respects except extremely low in zinc content, specific pathological changes were noted in the oesophagus, and to a lesser extent in the buccal cavity and the skin, and in a few animals, in the cornea.

The oesophagus showed extreme parakeratosis with a thick layer of partially keratinized cells.

The skin showed hyperkeratinization, thickening of the epidermis and loss of hair follicles with persistence of the sebaceous glands. Secondary bacterial infection occurred later.

The cornea of two animals showed vascularization and leucocytic infiltration similar to that which has been described in riboflavin deficiency. These ocular changes may indicate that zinc deficiency impairs the absorption or utilization of riboflavin.

The technical work was performed by Miss Miriam C. Reed.

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PLATE 1

EXPLANATION OF FIGURES

Photographs by Mr. Milton Kougl

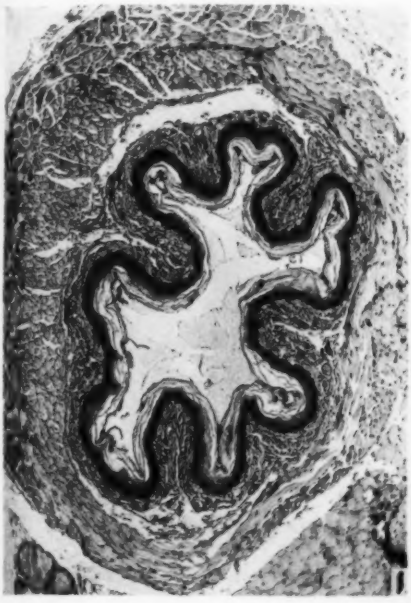
1 Low power photomicrograph of normal oesophagus.

2 Lower power photomicrograph of oesophagus from Zn-deficient rat. Same magnification as figure 1.

3 Higher power photomicrograph of normal lining of oesophagus.

4 Higher power photomicrograph of lining of oesophagus of Zn-deficient animal. Same magnification as figure 3.

5 Photomicrograph of cornea of one of the two animals showing vascularization with leucocytic infiltration.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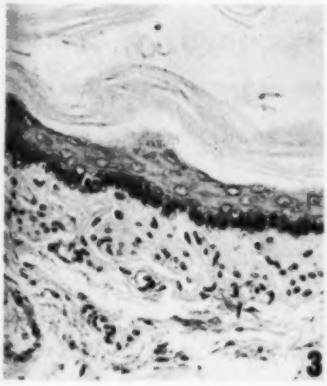
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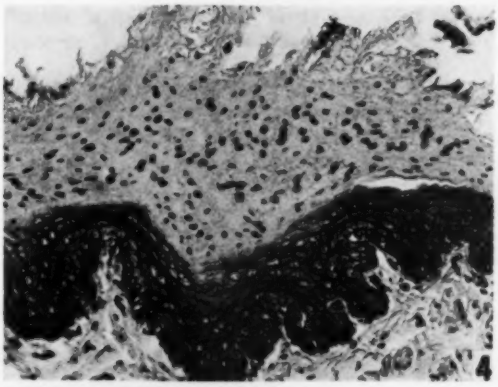
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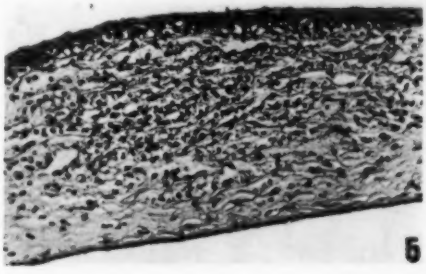
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PLATE 2

EXPLANATION OF FIGURES

6 Photomicrograph of skin of normal rat showing hair follicles and small sebaceous glands.

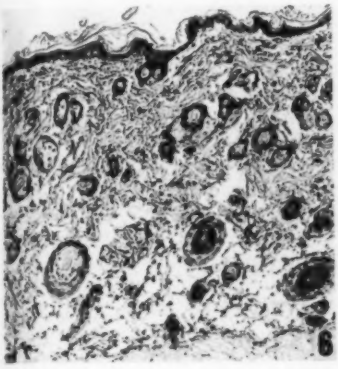
7 Photomicrograph of skin of Zn-deficient rat to show absence of hair follicles, enlargement of sebaceous glands, and thickening of epidermis.

8 Higher power photomicrograph of normal skin.

9 Higher power photomicrograph (same magnification as fig. 8) of Zn-deficient rat's epithelium.

10 High power photomicrograph of Zn-deficient rat's skin to show intercellular oedema and leucocytic infiltration.

11 Photomicrograph of skin of nose of Zn-deficient rat to show keratinization and crust formation.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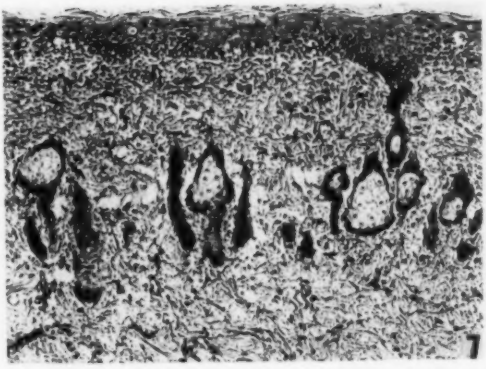
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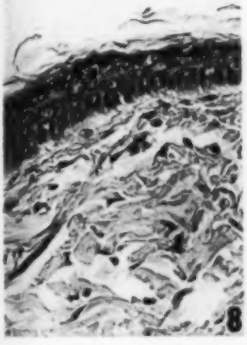
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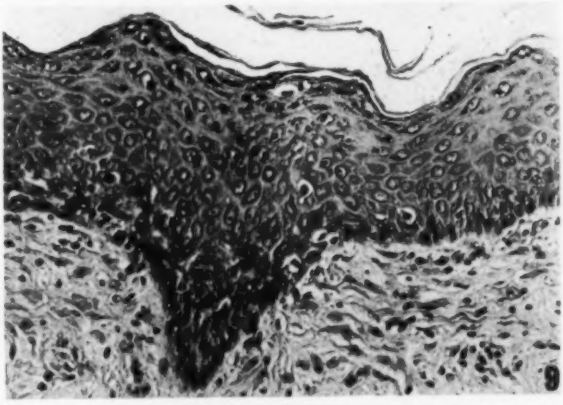
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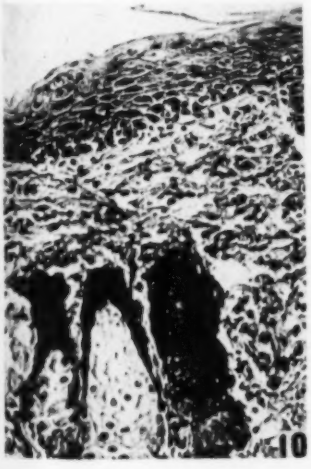
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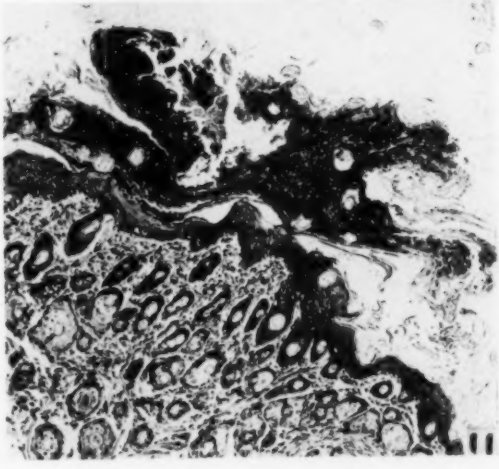
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EDITORIAL REVIEW

THE NUTRITIONAL IMPORTANCE OF CHOLINE [1]

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Relation of choline and of protein to liver lipids in older rats

The effects of choline deficiency which were recognized first and which, therefore, have been studied most extensively involve changes in the distribution of lipids in the animal body. As a primary result of the absence of dietary choline and of its precursors, neutral fat accumulates in the liver of the depancreatized dog and of the intact rat. The production of a "fatty liver" in the rat and its prevention by the inclusion of lecithin or of choline in the food mixture were reported by Best, Hershey and Huntsman ('32 a and b) following the demonstration, in depancreatized dogs, of the lipotropic action of raw pancreas (Allan et al., '24) and of lecithin (Hershey, '30; Hershey and Soskin, '31; Best and Hershey, '32). Best and Huntsman noted the choline-like action of betaine ('32) and the effectiveness of choline in

[1] This review has been limited purposely to the evidence of the dietary indispensability of choline, to its relation to other constituents of food mixtures and to the implications of its functions in metabolism. It has not been possible to include the extensive literature dealing with the activity of analogues of choline and of related chemical compounds (see Best and Ridout, '39) or with the significance of acetylcholine in myoneural physiology (see Alles, '34). Brief reference only has been made to the relation of choline to the transportation and utilization of lipids (see Best and Ridout, '39; Bloor, '40) and to its relation to the pancreatic factor, lipocaic (see Dragstedt, '40).

causing the disappearance of fat previously deposited in the livers of rats fed the low choline diet ('35). In these experiments fatty livers were produced in 3 weeks in 150 to 250 gm. rats on a mixed grain ration containing added fat.

The lipotropic action of casein was recognized by Best and Huntsman ('35) in experiments in which rats were first fed the above-mentioned high fat, low choline diet and then fed sucrose or sucrose and casein (20%). An additional deposition of liver fat occurred in the animals fed sucrose but not in those fed sucrose and casein. Channon and Wilkinson ('35) confirmed this effect of casein and introduced the low casein (5%), high fat (40%) diet which was widely adopted in the study of choline. The choline-like action of 1 gm. of casein was reported equivalent to 5 to 6 mg. of choline by Best, Grant and Ridout ('36) and to 7 to 8 mg. of choline by Beeston, Channon et al. ('36).

The addition of cholesterol to the diet of rats results in a "cholesterol fatty liver" in which there is a marked increase in cholesterol as well as in glycerol esters (Blatherwick et al., '33; Okey et al., '34). Best, Channon and Ridout ('34) demonstrated that supplements of choline in diets containing cholesterol completely prevent the deposition of glycerides but only partially prevent the accumulation of cholesteryl esters. Similar effects resulted from increases in the level of dietary casein (Beeston et al., '35; Best and Ridout, '36).

Synthesis of fat occurs in rats on low choline low fat rations (Longenecker et al., '40) and there is no evidence that the rate of oxidation of fat is seriously impaired by the lack of choline (Deuel et al., '37). The accumulation of glycerides in the liver probably results from a failure of transportation of fatty acids from this organ due to an inadequate supply of the choline required for the synthesis of the choline phospholipids. Although the phospholipids are generally believed to play an important part in the transportation of fatty acids (Sinclair, '34; Bloor, '40), the total amount of these phosphorylated compounds in liver remains relatively constant if calculated on a fat-free, dry weight

basis (MacLachlan and Hodge, '39). This constancy is also emphasized by Chaikoff who has demonstrated a marked stimulation of phospholipid "turnover" in the livers of rats following the administration of choline (Perlman and Chaikoff, '39 a and b). In view of the fact that a continuous supply of new choline appears to be essential for the movement of fatty acids out of the liver, it seems evident that a part of the choline used in such phospholipids is catabolized and does not return to the liver. No such direct explanation is acceptable, as yet, for the accumulation of cholesterol esters. Evidence which will be cited later points to the existence of other factors in addition to choline which are concerned with the metabolism of cholesterol.

Relation of choline, cystine and methionine to liver lipids in older rats

In order to determine which, if any, of the amino acids of casein might be responsible for the marked lipotropic activity of this protein, Beeston and Channon ('36) initiated the supplementation of the low casein ration with amino acids and found that cystine produces a surprising increase in the deposition of liver fat. In this connection, it is of interest that dietary cystine had previously been found to produce fatty degeneration of the liver in experiments which were not at the time related to choline metabolism (Curtis and Newburgh, '27; Lillie, '32). Tucker and Eckstein ('37) confirmed the results of Beeston and Channon and demonstrated that methionine exerts an opposite effect and is definitely lipotropic. Tucker et al. ('40) later found that if a 5% casein or edestin diet was supplemented with the amounts of methionine and cystine present in a 20% casein diet, the resulting lipotropic activity equalled that of the casein diet. This effect of methionine was confirmed by Channon, Manifold and Platt ('38) and by Best and Ridout ('40) although the latter questioned the conclusion that methionine was solely responsible for the lipotropic activity of casein. It is pertinent to note that in many of these experiments the dietary protein

was deficient in amount and in quality, a point recognized by Channon, Loach et al. ('38). It will be shown later that the symptoms of choline deficiency in young rats are affected by the nutritive state and that the cystine effect may only be a manifestation of an existing shortage of cystine or of total sulfur amino acids as well as of choline. Channon et al. ('40) have reported that amounts of cystine in excess of 4 mg. per day are no more effective than the 4 mg. level in causing an increased deposition of liver fat, an observation which suggests that the cystine effect may not be due to the specific interrelationship of cystine and choline.

Relation of choline to methylation of homocysteine

Additional evidence of the metabolic interrelationship of choline and of methionine is afforded by the investigations of du Vigneaud and his coworkers who demonstrated that supplements of choline or betaine in a diet which contained homocystine but which was devoid of methionine and cystine stimulated the growth of young rats (du Vigneaud, Chandler et al., '39). In the earlier experiments of White and Beach ('37) homocystine improved a low methionine diet and these workers suggested the conversion of homocystine into methionine. Later, du Vigneaud, Dyer and Kies ('39) reported that homocystine permitted growth on a diet otherwise devoid of sulfur amino acids if tikitiki and milk vitamin concentrate were used as sources of the vitamin B complex but not if a mixture of pure vitamins was supplied, and Rose and Rice ('39) reported similar results. Having noted that fatty livers occurred in his experimental animals, du Vigneaud added choline to the purified vitamin supplement and demonstrated, as cited above, that homocystine and choline may be substituted for methionine (and cystine). The study by du Vigneaud, Chandler et al. ('39) demonstrated the presence of choline in the material used as a source of the B complex in the experiments in which homocystine had been found to stimulate growth. In subsequent experiments du Vigneaud et al. ('40) showed by means of isotopic hydrogen that this element,

which was administered to rats in the methyl group of methionine, could be found later in tissue choline and creatine. As a result of these remarkable observations, du Vigneaud concluded that transmethylation, the specific transfer of methyl groups, occurs in the animal organism; that the methyls of methionine, choline and betaine are transferable; and, that methyl groups in such utilizable forms may be essential in the diet. Welch ('41) has reported the preparation of a methionine-free hydrolysate of casein as a substitute for the mixture of pure amino acids used by Rose and by du Vigneaud in the study of the metabolism of homocystine.

Relation of choline to hemorrhagic degeneration in young rats

The importance of dietary choline is further emphasized by the investigations of Griffith et al., who described an acute pathological state, hemorrhagic degeneration, in young male rats on diets which appeared adequate in protein and in dietary essentials other than choline. On these diets, rats, 21 to 26 days of age, developed fatty livers within 48 hours and severe hemorrhagic renal degeneration, ocular hemorrhage and regression of the thymus within 10 days (Griffith and Wade, '39 and '40; Griffith, '40 b). The results were spectacular not only because of the rapid onset of these effects but also because of the equally rapid "recovery" occurring in those rats which survived. The renal hemorrhage disappeared in the recovery phase but the fatty liver and signs of renal pathology persisted. With the exception of the new manifestations of the lack of choline, the metabolic aspects of this deficiency in young rats resembled those involved in the occurrence of fatty livers in older rats. Hemorrhagic degeneration was prevented by choline, methionine and betaine (Griffith, '41; Griffith and Mulford, '41 a) and was aggravated by cystine and cholesterol (Griffith, '40 a; Griffith and Mulford, '41 b). It was evident in these experiments that the choline requirement of young growing rats is greater than that of older animals since the severe effects appeared on

diets containing amounts of casein (15 to 25%) which are protective if fed to older animals.

The severe renal pathology occurring in young rats on rations deficient in choline (and low in methionine) had apparently been noted previously but had not been correlated with the lack of choline (Hartwell, '28; Cox et al., '29; Cox and Hudson, '30). Discoloration of the kidneys was observed by du Vigneaud, Dyer and Kies ('39) and du Vigneaud, Chandler et al. ('39) reported renal hemorrhage in one rat on the diet containing homocystine as the source of the sulfur amino acids. Inasmuch as the 18% casein diet does not supply sufficient methionine to prevent choline deficiency, it may appear surprising that the renal effects have not been noted more frequently in nutritional investigations. It is of interest in this connection that the deficiency is less severe in rats of both sexes if over 30 days of age and in female rats less than 30 days of age (Griffith, '40 b) and that severe renal hemorrhage has not been observed, even in young male rats, if the sucrose of the basal ration is replaced by starch (unpublished experiments). An additional finding, which is believed to be of considerable importance in the interpretation of results concerned with the requirement of choline, is the fact that the renal pathology and fatty liver do not occur if there is restriction of the intake of a food mixture which produces severe damage when fed ad libitum.

This direct relation between the rate of metabolism or of growth and the need of choline is also believed to be the basis for the apparent antagonism between this substance and cystine. In seeking an explanation for the aggravating effect of supplements of cystine in the 18% casein diet and particularly for the fact that 1% of added cystine was no more injurious than 0.3%, the suggestion was made that 18% casein is an inadequate source of sulfur amino acids, that added cystine prevents cystine deficiency, and that extra choline is then required because of the improved nutritional state (Griffith, '41). Definite evidence is now available (Mulford and Griffith, unpublished experiments) that the 18%

casein diet which is supplemented with choline is significantly improved by the addition of cystine. This improvement is characterized by greater increases in the body weight and length of the experimental rats and particularly by an increased efficiency of utilization of food.

Relation of choline to thiamine and other vitamins of the B complex

Supplements of thiamine in low choline diets increased the deposition of liver fat in rats, and McHenry ('37 a and b) concluded that this vitamin and choline are antagonistic and that both are required for the normal utilization of fats. Gavin and McHenry ('40) noted that supplementary pyridoxine (B_6) and nicotinic acid slightly augment the body fat of rats on low-choline diets but that neither these two vitamins nor riboflavin prevent the deposition of liver fat which results from the administration of thiamine. The amount of liver fat was normal if choline was administered, either alone or with any combination of the three vitamins. Halliday ('38) reported that pyridoxine deficiency in rats aggravated the effect of choline deficiency with respect to deposition of liver fat and Gyorgy and Goldblatt ('40) found that renal hemorrhagic lesions occurred more frequently if the food mixture contained pyridoxine.

Griffith and Mulford ('41 b) were unable to demonstrate any effect of supplements of thiamine or of pyridoxine on liver fat in experiments in which these compounds were added to a diet which contained yeast and which was adequate for growth. The previously cited protective effect of subnormal metabolism induced by restriction of food intake raises the question as to whether the results of McHenry and of Gyorgy really represent direct antagonisms of thiamine and of pyridoxine to choline. Dietary supplements which improve deficient or even suboptimal diets may appear to act in direct opposition to choline. Such an effect may be nonspecific and have no direct relation to the metabolism of choline itself.

Relation of choline to phospholipid turnover in the liver

Chaikoff has used radioactive phosphorus (P^{32}) to advantage in the study of the metabolism of the phospholipids. Choline accelerates the formation and removal of phospholipids in rat livers (Perlman and Chaikoff, '39 a). This effect is evident within 1 hour and is proportional to the amount of choline administered. The phospholipid turnover is also stimulated by betaine (Perlman and Chaikoff, '39 b) and by cystine and methionine (Perlman et al., '40 a and b). Cholesterol markedly decreases the turnover and the stimulating effect of choline is more evident in cholesterol-fed than in fat-fed rats (Perlman and Chaikoff, '39 c). Fries et al. ('40) studied the effect of age on phospholipid turnover in the central nervous system of rats and found that the activity is maximal on the first day of life and that it decreases to a low level by the time the rat weighs 50 gm. This is of interest in view of the fact that Griffith ('40 b) found a marked decrease in the choline requirement of rats over 30–35 days of age. Chanutin ('31) has concluded that young rats reach "chemical maturity" between 30 and 40 days of age.

Relation of choline to manganese in the prevention of perosis in birds

The role of manganese in the prevention of perosis (slipped tendon disease) was indicated by the observations of Wilgus et al. ('36) and Insko et al. ('38). Later, Hogan et al. ('40) reported that perosis, which occurred in spite of adequate dietary manganese, was prevented by some constituent of an alcoholic extract of liver. Jukes ('40 a and b, '41) found that choline prevented perosis and stimulated the growth of young turkeys and chicks on rations supplemented with manganese and that betaine was ineffective as a substitute for choline in such experiments. The antiperotic action of choline was confirmed by Hogan et al. ('41) and by Hegsted et al. ('41).

Other effects of choline

The administration of choline depresses hematopoiesis, induced by cobalt, in dogs (Davis, '39); prevents papillomatous lesions in the forestomach of rats on a diet containing 89% white flour (Sharpless, '40); is indispensable for lactation in adult rats and for growth and prevention of paralysis in suckling rats (Sure, '40); and, is essential for normal nutrition and egg production in chickens (Abbott and DeMasters, '40). Bloor ('40) has emphasized the relation of choline and of lecithin to acid-base equilibria and to the excretion of phosphate by the kidneys.

Relation of choline to unidentified dietary factors concerned with the metabolism and transport of lipids

The complexities of lipid metabolism are only partly clarified by the demonstrated effects of dietary choline. Evidence is increasing for the existence of one or more additional compounds which have a role in the choline-lipid interrelationship. Some of this evidence is as follows: on certain basal diets, supplements of methionine and cystine are not equal in lipotropic activity to equivalent amounts of these acids supplied as casein (Best and Ridout, '40); the accumulation of cholesterol esters is only partially prevented by supplements of choline in diets containing cholesterol (Blatherwick et al., '33); raw pancreas is more effective than choline in preventing the decrease in the lipids of the blood of dogs after ligation of the pancreatic ducts (Entenman et al., '39, '40 and '41); the accumulation of liver lipids induced by an aqueous extract of liver is not prevented by choline (Blatherwick et al., '33) but is prevented by lipocaic (McHenry and Gavin, '40); a pancreatic fraction, lipocaic may be more effective than choline in the prevention of fatty degeneration of the liver (Dragstedt, '40); and, renal and ocular hemorrhage occur in rats after 30 to 40 days on a diet containing cholesterol and choline even though protection is afforded during the crucial 6 to 10 day period (Griffith

and Mulford, '41 b). In the latter experiments, the similarity in the hemorrhagic lesions found at the end of the shorter period in the absence of choline and at the end of the longer period in the presence of choline suggests that these effects are not due to the lack of labile methyl alone. It may be that choline serves as one precursor of the lecithin required for the prevention of the fatty liver and, in addition, is used as choline, or as a source of methyl, in the synthesis of an unrecognized compound needed particularly in the metabolism of cholesterol. Such a compound, or its precursor, might be supplied by the diet and by the tissues of the rat in sufficient amounts during the 6 to 10 day period on a diet containing choline but not during a 30 to 40 day period. It is noteworthy that the later occurrence of renal hemorrhage on the cholesterol diet containing choline was not associated with marked deposition of liver lipids.

The choline-like action of methionine and betaine appears satisfactorily explained on the basis of a transfer of methyl groups by the process of transmethylation proposed by du Vigneaud. Other compounds having monomethyl and even trimethyl (betaine) groups attached to nitrogen are known to occur in animal tissues. The metabolic significance of these substances, ergothioneine and carnitine, for instance, remains to be determined.

The daily requirement of choline in young rats

Best and Huntsman ('35) proposed that choline be considered a dietary essential. Such a conclusion is justifiable even though signs of choline deficiency may be prevented by methionine or by betaine and even though synthesis of choline may occur in the rat (Jacobi et al., '41). The demonstrated value of dietary choline in rats and chicks is evidence of the inability of the organism to supply its own choline in adequate amounts by synthesis. Excessive levels of even high methionine proteins, such as casein, are necessary if sufficient methionine is to be supplied in the form of protein. Betaine is less effective than choline in the prevention of hemorrhagic

degeneration (Griffith and Mulford, '41 a) and as a supplement in a diet containing homocystine as the sole sulfur amino acid (Chandler and du Vigneaud, '40). Although it is logical to believe that choline is needed as such in view of its occurrence in the choline phospholipids, it is probable that it also serves in normal metabolism as a source of labile methyl in the synthesis of other important compounds.

The daily requirement of choline may be stated with respect to a specific food mixture only, since it varies with the dietary methionine, cystine, betaine and cholesterol as well as with the adequacy of the ration for optimum growth. The requirement has been determined by Griffith and Mulford (unpublished experiments) in male rats, 40 gm. in weight and 21 to 26 days of age, fed a basal diet with the following percentage composition: casein 6 to 42, lard 19, inorganic salts 5, agar 2, yeast 6, fortified fish liver oil 1 and sucrose 25 to 61. Very high casein levels of 30% or more are necessary to supply sufficient methionine to permit omission of a choline supplement. On casein levels of 18 to 24%, hemorrhagic degeneration is prevented by 1 to 2 mg. of choline chloride daily and the deposition of liver lipids by 4 to 6 mg.

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REDUCTION IN EXPERIMENTAL RAT CARIES BY FLUORINE [1]

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SIX FIGURES

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Two recent independent observations on commercial casein as a dietary constituent are of interest: (a) that the commercial casein on the market sometime ago produced dental fluorosis in rats and was shown to contain about 0.2% of fluorine (Hodge, Luce-Clausen and Brown, '38), and (b) that commercial casein provided a protective action against experimental caries in rats (Lilly, '38). Combining these observations, the hypothesis was set up that fluorine was the factor supplying the protection. This hypothesis was the more tenable since both chemical analyses (Armstrong and Brekhus, '38), and clinical surveys (Dean et al., '39), had pointed to a relationship between caries activity and fluorine. Consequently groups of rats were fed diets containing "fluorine-free" casein with and without the addition of fluorides. Since this experiment was begun two reports have appeared (Miller, '38; Cox et al., '39), which demonstrated that fluorine inhibits experimental caries in rats. The data presented herewith not only confirm these findings but amplify those of Hodge and Finn ('39), and extend the previous observations.

[1] This work was supported in part by a grant from the Carnegie Corporation of New York. The data in this paper were taken from the thesis submitted by the senior author to the Committee on Graduate Studies of the University of Rochester in partial fulfillment of the requirements for the degree Master of Science.

EXPERIMENTAL

One hundred and thirty-five rats (Wistar strain) were divided into three groups at weaning. The first group received the Hoppert, Webber and Canniff ('31) caries-producing diet,[2] the second group received the same diet with the substitution of commercial casein for the powdered whole milk (Lilly, '38), the third group received the latter diet with the addition of 3 mg. of fluorine (as KF) daily. The fluoride was given by pipette in a drop of water deposited on the tongue. The amount of fluorine is the same as would have been received had the dietary casein contained 0.2% fluorine. Each group of rats was subdivided into three groups; these received respectively, (a) corn particles (cracked corn) larger than 20 mesh only, (b) corn particles between 10 and 20 mesh, and (c) unsifted or run-of-the-mill cracked corn. The diets and distilled water were given ad libitum.

The rats were weighed every 4 days. The teeth were examined periodically using H. R. Hunt's technic (personal communication) for the evidence of carious destruction. Radiographs of the tibias on the fiftieth day using the technic of O'Brien and Morgareidge ('38) showed rickets in the rats receiving casein. This disease was promptly healed[3] (as shown by radiographs on the sixtieth day) through the incorporation of 2% whole yeast and 2% cod liver oil into the diets.

After 200 days on the diets, the rats were sacrificed, the jaws separated and studied in three ways. (1) Using a binocular microscope (15 ×) the jaws were examined occlusally for caries and for fractures. (2) Through the kindness of Dr. G. J. Cox, the method of Cox and Dixon ('39) was applied to all the right jaws. This procedure detects and grades fissure caries by repeated successive grinding, staining and examination with reflected light (15 ×). (3) The left jaws were prepared as ground and decalcified stained sections to observe

[2] Coarse corn meal 60%, powdered whole milk 30%, linseed meal 6%, alfalfa meal 3%, NaCl 1%.

[3] For radiographic details of the effect of fluorine on rickets, see Morgareidge and Finn ('40).

the nature and progress of the lesions. The results of these histological studies are to be presented elsewhere. In brief, these examinations gave indications as to the nature of rat caries but shed little light on the effect of fluorine.

RESULTS

In figure 1 are given the data for the total incidence of caries in the molar teeth of the various groups of rats. The data are presented separately for each tooth type, e.g., first molar on the right side of the maxilla, et cet. It is evident (1) that first molars (both upper and lower) have a higher incidence of

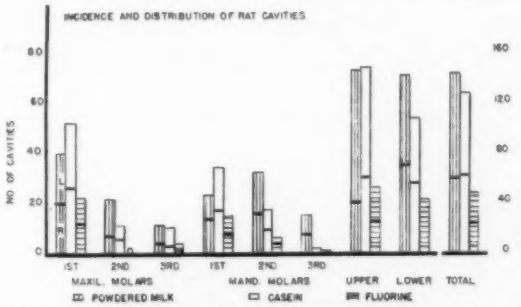

Fig. 1 The number of cavities is given as the ordinate; the data for each tooth type are given separately. The totals for the left side of the jaws are represented by the height above the horizontal dividing lines in each column, those for the right side by the lower part of each column. For each tooth, for the lower and upper jaws, and for the over-all totals, the fluorine-fed group had a lower caries incidence.

caries than the other molars although no molar type was completely caries-free; and (2) that for each tooth the total number of cavities was similar for the whole powdered milk and the casein fed groups but that a significant decrease in cavities was found in the group receiving fluorides. This fact is also demonstrated in the totals for upper and lower jaws and the over-all totals given on the right side of figure 1. The reduction in the number of cavities by fluorine (table 1) is shown to have a high probability of significance (table 2). The teeth of the upper jaw had more cavities (171) than those of the

TABLE 1

Caries incidence for the various dietary groups and sub-groups.

DIETARY SUPPLEMENT	CORN PARTICLE SIZE	MESH	NO. RATS	NO. OF CAVITIES		NO. OF CUSPS INVOLVED		NO. OF TEETH DESTROYED		NO. CARIES-FREE MOUTHS
				total	*per rat*	*total*	*per rat*	*total*	*per rat*	
Powdered whole milk (30%)	Coarse	20	14	48	3.4	171	12	14	1.0	0
	Fine	10–20	13	55	4.2	143	11	10	0.8	0
	Run of mill	Unsifted	13	39	3.0	111	8	10	0.8	0
	Totals and averages		40	142	3.5	425	11	34	0.9	0
Casein (30%)	Coarse	20	12	39	3.3	105	9	5	0.4	0
	Fine	10–20	15	45	3.1	147	10	10	0.7	0
	Run of mill	Unsifted	15	42	2.8	117	8	6	0.4	0
	Totals and averages		42	126	3.0	369	9	21	0.5	0
Casein (30%) and fluorine (3 mg.)	Coarse	20	13	12	0.9	40	3	1	0.1	5
	Fine	10–20	14	19	1.3	50	4	1	0.1	3
	Run of mill	Unsifted	15	16	1.0	42	3	3	0.2	5
	Totals and averages		42	47	1.1	132	3	5	0.1	13

TABLE 2

Statistical analysis of data of table 1.

	POWDERED MILK	CASEIN	FLUORINE
No. cavities	142	126	47
No. cavities per rat	3.6	3.0	1.1
Compared to F group			
s[1]	1.23	1.06	
s_{-x}	0.27	0.23	
t	9.2	8.2	
N	80	82	
P	0.01	0.01	
No. cusps involved	425	369	132
% total cusps	19.0	15.7	5.6
Significance ratio[2] of mean differences with F group	14.1	11.4	
No. teeth destroyed	34	21	5
% total teeth	7.1	4.4	1.1
Significance ratio of mean differences with F group	5.0	3.1	
No. caries free mouths	0	0	13
% total mouths	0	0	31
Significance ratio of mean differences with F group	4.3	4.3	

[1] The statistical procedure used is given in Fisher ('34).

[2] The statistical procedure used is given in Yule and Kendall ('37). The "significance ratio" is equal to the actual difference in average percentages divided by the standard error of sampling.

lower jaw (145); calculations indicated that this difference would occur by chance twice out of ten trials. There was no regular tendency for an asymmetrical distribution of caries (right vs. left sides of the jaw).

The number of carious cusps for the various teeth is given in figure 2. The maxillary first molar is most frequently involved and, as before, there is evidence that the teeth of the upper jaws are more often carious. The reduction in the number of carious cusps in the rats receiving fluorine is clearly shown in the column for total values at the right. The probability that such a difference would occur by chance is very low (table 2).

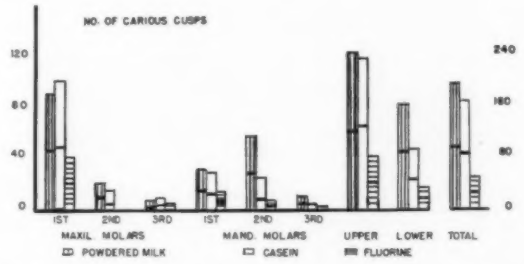

Fig. 2 The plotting is done as in figure 1. The reduction in the number of carious cusps of the fluorine-fed group is evident for each tooth type, for upper and lower jaws and for the over-all total.

Apparently, not only is the maxillary first molar the most frequent site of carious attack but the number of these teeth completely destroyed nearly equals the total for all the other molar types (fig. 3). What is meant by "completely destroyed" may be seen in figure 4; the crown of a mandibular second molar has been involved and has disappeared allowing the third molar to drift forward. The traumatic changes following the massive destruction of a tooth inevitably spread to the jaw bone as can be seen in the radiograph, figure 5. The inhibiting effect of fluorine is shown in the total number of teeth destroyed for the various groups, viz., milk—34, casein—21, fluorine—5.

The reduction by fluorine has a high statistical significance (table 2). Varying the particle size of the cracked corn had no significant effect on the caries incidence (table 1) whether the data are considered for number of cavities, number of teeth destroyed or number of cusps involved. It should be noted that all of the diets contained corn particles at least as large as 10 to 20 mesh, i.e., "coarse" corn particles.

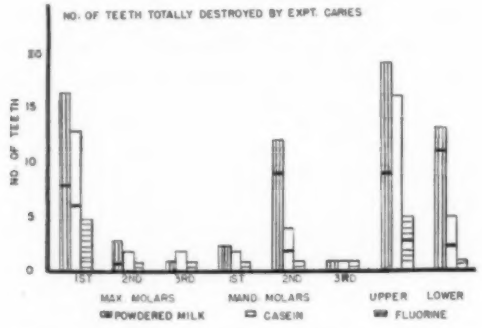

Fig. 3 The plotting is done as in figure 1. Note the very high destruction of maxillary first molars as well as the reduction of teeth destroyed in each case when fluorine was added to the diet.

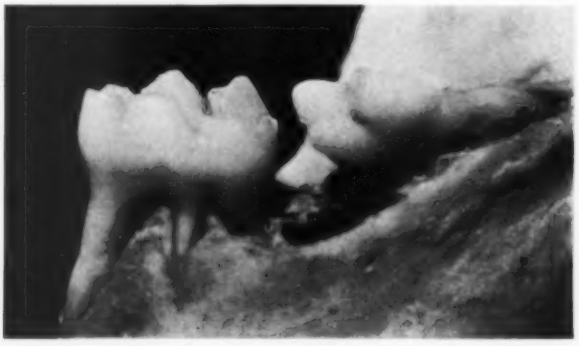

Fig. 4 "Complete destruction" of a mandibular second molar. The third molar has drifted forward. Note the sharp cusps of the first molar and the evidence of fracture of the cusp nearest the second molar. (10 ×, photograph by M. C. Orser.)

The growth curves for the male and female rats (fig. 6) show that the casein and fluorine groups lagged a little behind the milk-fed group in body weight; this may in part be due to the deficiency already described which was corrected on the

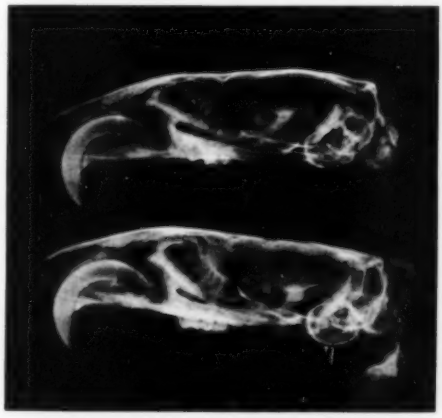

Fig. 5 Rat skulls are separated longitudinally into halves by the technic of B. J. Frey (personal communication) and radiographed. The upper skull shows the complete destruction of the first molar and the concomitant involvement of the adjacent alveolar bone. The lower skull is from a normal rat. (Print by P. S. S. Sweet, Eastman Kodak Co.)

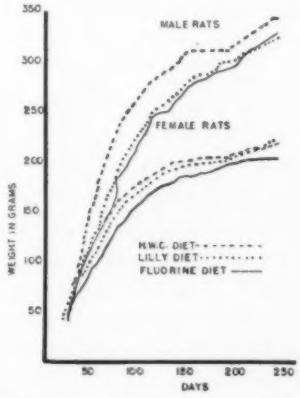

Fig. 6 Composite growth curves of the male and female rats on the diets indicated. The fluorine-fed rats grew well and gave no indication of a pronounced toxic effect.

fiftieth day. In general, the rats grew well, the fluorine-fed group giving no indication of a toxic effect on growth. The earliest indication of macroscopic caries was observed between the forty-fourth and fiftieth day on the diet. The seventy-two male rats showed the same caries incidence as the fifty-two female rats; these comparisons were made group by group.

The results of the examination of the teeth for fissure caries (through the cooperation of G. J. Cox) were surprising. In only seven teeth, four of which were lower first molars, were suspicious areas found. Of these, four were probably artifacts and only three could be classed as true fissure caries. Perhaps the long duration of the experiment permitted other fissure caries to coalesce with or simulate occlusal cavities and thus prevent identification.

Summarizing the effects (table 1) attributed to the fluorine in the diet: (1) there were on the average 1.1 cavities per rat fed fluorine; 3.5 per rat not fed fluorine. (2) Rats fed fluorine averaged 3 cusps involved per rat; the other two groups averaged 11 and 9 per rat. (3) On the average the fluorine-fed rats lost 0.1 tooth per rat; the other groups lost 0.9 and 0.5 tooth per rat. It is noteworthy that thirteen of the forty-two rats receiving fluorine had caries-free mouths; by sharp contrast, none of the eighty-two rats in the other groups had a caries-free mouth. This difference is statistically significant (table 2).

DISCUSSION

It may almost be said that every element known to exist in dental tissues has at one time been suspected as the cause of dental caries. However, various authors have differed in ascribing the disease on the one hand to an excess and on the other to a deficiency of the element in question. Fluorine is no exception; Sir Crichton-Browne (1892) suggested that a fluorine deficit might be the causal agent since, on the basis of earlier analyses, teeth were shown to be comparatively rich in fluorine. During the next decade Wrampelmeyer (1893) and Hempel and Scheffler (1899) reported higher fluorine values in sound than in carious teeth; in the light of present knowl-

edge the high method error invalidates their data. Only recently was a fluorine method of proved accuracy developed and a significantly higher fluorine content established for the enamel of sound teeth by Armstrong ('37) and Armstrong and Brekhus ('38). From their data the need of precise information is emphasized by two points. First, minute quantities of fluorine are present in tooth tissues. A 2-gm. tooth has perhaps 700 mg. of enamel; of this only 0.01% is fluorine or 70 μg. Second, the enamel of a sound 2-gm. tooth would contain on the average about 30 μg. more fluorine than would be found in the enamel of a carious tooth of the same weight.

There is evidence from the data of table 1 that fluorine limits caries progress as well as reduces the incidence of lesions. Fluorine-fed rats developed almost half as many cavities as the casein-fed controls (1:2.7), but the size of the cavities was less. Thus, for each cusp involved in the fluorine group, there were 3.6 cusps involved in the control group. The limitation of caries extension is further shown by the relative numbers of teeth destroyed; for each tooth destroyed in the fluorine-fed group, 4.2 teeth of the controls were destroyed. In the occlusal examination, fractured cusps which showed no concomitant staining or other signs of carious attack were not counted as cavities. There was little, if any, qualitative difference in the appearance of fractures in the teeth of the fluorine-fed group as compared to the controls; all the rats exhibited many fractured cusps. The data given above lead to the hypothesis (1) that fluorine prevents the development of a carious lesion on the site of a fractured cusp, thus giving the caries-free mouths; and (2) that fluorine prevents the development of a small cavity into a larger one, thus giving the lesser number of cusps involved and whole teeth destroyed.

While the experiment reported in this paper was being carried out, Miller ('38) reported that fluorides or iodoacetate added to the diet of rats produced a marked reduction in experimental caries. The data he presented from the lower molar teeth of fifty rats fed for 100 days on caries producing

diet showed that calcium fluoride gave an 81% reduction, sodium fluoride 94% and iodoacetic acid 100% reduction in caries incidence. That only 70% reduction is shown in table 1 may in part be attributed to the longer duration of this experiment (200 days) and in part to the inclusion of both upper and lower molars in the totals. However, in general, these results confirm the work of Miller ('38). Cox and co-workers ('39) feeding much less fluorine (20 p.p.m. of the diet) than Miller and ourselves did (ca. 300 p.p.m.) showed statistical evidence of a reduction in caries less dramatic than the reduction shown in table 1 or by Miller ('38).

It is difficult to make even tentative statements about the site of fluorine action or the mechanism of the inhibition of experimental caries. Rat caries as a phenomenon is not perfectly understood; there has been a long controversy over the manner of initiation and progress of the lesions, especially as to whether the enamel is the initial site of attack. If enamel caries is the first step then surface phenomena, such as the absorption of fluorides as shown by Volker et al. ('40), and repression of bacterial acid production as shown by Bibby and Van Kesteren ('40) would assume prime importance. If the initial lesion is a cusp fracture thereby exposing dentine and the subsequent course is a true dentinal caries, then in addition to the above-mentioned factors, the systemic and metabolic effects of fluorine and the defense mechanisms of the dentine would be involved. Volker ('39) has demonstrated not only that the lactic acid solubility of powdered normal enamel and dentine is reduced by a preliminary treatment with a solution of fluorides, but also that the enamel and dentine from rats fed fluorides were less soluble than these tissues from normal rats. The idea that solubility reduction and bacterial enzyme inhibition are major factors in the reduction of experimental rat caries must at present be regarded as conjecture; the mechanism of the inhibiting or protective action of fluorine is unknown.

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THE ASCORBIC ACID CONTENT OF COW'S MILK DURING PREGNANCY

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The ascorbic acid content of cow's milk has been given considerable attention since Tillmans, Hirsch and Hirsch ('32), Harris and Ray ('35) and Bessey and King ('33) introduced reliable and rapid methods employing 2, 6-dichlorophenolindophenol for the determination of this factor in biological materials. However, a thorough survey of available literature failed to reveal any information concerning the influence of pregnancy on the amount of ascorbic acid in cow's milk. Therefore this study was conducted to determine the relationship, if any, between this physiologic state and the vitamin C content of the milk of a group of nineteen Guernsey and Holstein cows.

METHODS

The age of the Guernseys varied from 4 to 8 years and the Holsteins from 5 to 9 years. The animals were housed in a modern dairy barn, were stall-fed, and throughout the experiment had no access to pasture. All cows were under the observation of an experienced veterinarian and were maintained for the production of certified milk under carefully controlled conditions on a Massachusetts farm. All the experimental animals were negative to the agglutination test for Brucella Infection, and mastitis was controlled by physical and laboratory tests together with approved sanitary practices.

The attendants were under constant medical supervision in order to eliminate human infections or milk-borne diseases.

The cows received a daily ration compounded from various combinations of the following ingredients: first and second grades of mixed hay, first and second grades of alfalfa hay, legume and grass silage, beet pulp, dairy ration, dairy ration with 6.7% irradiated yeast, home-mixed fitting ration with 6.7% irradiated yeast and a commercial fitting ration with irradiated yeast. The authors ('39, '40) have previously published data concerning the composition of the rations and the ascorbic acid content of their ingredients.

Samples of milk, which were assayed for their vitamin C content, were taken at bimonthly intervals from the morning milking (3:00 A.M.). The samples were collected in 30 cc. flint glass bottles which were immediately cooled, placed on ice and protected from light until assayed. The amount of ascorbic acid in the milk was determined by the usual indophenol titration method as described in a previous publication by Holmes and co-workers ('39). Since the authors ('39) found that the milk from Guernsey cows contained more vitamin C than that of Holstein cows, the results of the analyses (table 1) are reported separately for the two breeds in terms of milligrams of ascorbic acid per liter of milk. Table 1 also supplies data concerning the number of assays and the average vitamin C content of milk collected at different stages of pregnancy.

RESULTS

The smallest amount of vitamin C found in the milk of the Guernseys was in the ninth month of pregnancy when 17.88 mg. per liter was obtained in a single determination. This is essentially the same as 17.91 mg. per liter obtained for this group in the eighth month of pregnancy. The amount of ascorbic acid found in the milk of the Guernseys during the eighth month of pregnancy varied from 14.95 mg. per liter for cow no. 2 to 19.54 mg. per liter for cow no. 1.

The average figures for the ascorbic acid content of milk obtained for cows in the fifth month of pregnancy fell within

TABLE 1

Pregnancy and the ascorbic acid content of milk.
All values are in milligrams of ascorbic acid per liter.

COW NO.	MONTH OF PREGNANCY 1	2	3	4	5	6	7	8	9
Guernseys									
1	....	20.19	20.80	21.10	20.60	18.93	15.71	18.64	17.88
	....	19.50	19.70	19.10	20.31	18.70	19.69	19.54	....
2	....	22.09	23.13	20.53	16.24	18.98	19.51	14.95	....
	....	12.32	21.01	20.51	18.52	18.63	18.30	....	....
	....	....	22.67	....	....	....	....	....	....
3	19.63	20.51	20.42	21.59	19.55	18.36	19.36	18.51	....
	20.86	18.53	21.43	19.44	19.31	17.46	17.61	....	....
	....	20.48	....	....	....	....	....	....	....
4	....	....	....	....	21.37	21.17	19.55	....	....
	....	....	....	....	16.56	17.85	20.19	....	....
	....	....	....	....	....	24.30	....	....	....
5	25.48	....	....	....	....	....	....	....	....
6	22.62	21.59	21.09	17.82	....	....	....	....	....
	21.80	20.60	....	18.36	....	....	....	....	....
7	25.70	27.68	....	....	....	....	....	....	....
	27.03	22.74	....	....	....	....	....	....	....
8	23.02	21.66	21.75	22.93	....	....	....	....	....
	21.97	21.36	21.94	22.14	....	....	....	....	....
9	21.82	18.53	....	....	....	....	....	....	....
	21.16	18.52	....	....	....	....	....	....	....
10	18.40	18.19	13.97	19.88	16.86	21.08	....	....	....
	....	18.36	19.36	....	17.72	....	....	....	....
	....	....	19.33	....	....	....	....	....	....
11	21.44	21.80	22.87	20.43	17.46	....	....	....	....
	23.71	25.33	21.47	21.00	18.36	....	....	....	....
No. cows	8	9	7	7	6	5	4	3	1
No. det'ns.	14	19	15	13	12	10	8	4	1
Average	22.47	20.53	20.73	20.37	18.57	19.55	18.74	17.91	17.88
Holsteins									
12	16.41	15.78	13.41	9.84	16.02	....	....	....	....
	16.51	14.11	11.88	11.52	10.70	....	....	....	....
13	22.84	20.97	16.48	15.74	18.30	16.80	14.62	....	....
	18.68	....	16.71	16.41	....	16.86	....	....	....
	....	....	....	17.02	....	....	....	....	....
14	17.26	17.87	17.12	....	....	....	....	....	....
	15.43	16.83	....	....	....	....	....	....	....
15	....	....	....	....	20.12	19.41	....	....	....
	....	....	....	....	20.02	18.21	....	....	....
16	19.76	....	....	....	....	....	....	....	....
17	....	....	....	....	19.04	20.58	20.09	....	....
	....	....	....	....	19.25	20.08	....	....	....
18	14.34	15.45	16.56	19.34	18.23	16.90	17.57	15.64	....
	....	....	18.04	17.75	15.60	16.05	17.44	13.80	....
	....	....	....	18.25	....	....	....	....	....
19	16.34	16.73	18.25	12.35	19.70	16.61	16.10	....	....
	17.33	16.82	18.56	17.87	16.86	17.15	....	....	....
	15.68	....	....	....	....	....	....	....	....
No. cows	6	5	5	4	6	5	4	1	..
No. det'ns.	11	8	9	10	11	10	5	2	..
Average	17.33	16.82	16.33	15.61	17.62	17.87	17.16	14.72	..

the limits observed for the eighth month of pregnancy. The highest average vitamin C content of the group of Guernsey cows was 22.47 mg. of vitamin C per liter, for cows in the first month of pregnancy. However, cows in this first month also showed wide variations. Cow no. 10 produced milk with a vitamin C content of 18.40 mg. per liter while the milk from cow no. 7 contained 27.03 mg. per liter. The data for the Guernsey cows show that wide variations in vitamin C content of the milk occur at all stages of pregnancy. However, when the average figures are considered there is a gradual downward trend in the concentration of vitamin C as pregnancy progresses.

The data obtained for the milk from the Holstein cows show that the smallest average ascorbic acid content was 14.72 mg. of vitamin C per liter for the eighth month of pregnancy. The highest average value, 17.87 mg. per liter, was found in the milk of cows in the sixth month of pregnancy. However, this figure exceeds but slightly the amount found in the milk of the Holsteins during the first, fifth and seventh months of pregnancy. As in the case of the Guernsey cows, the milk from Holstein cows at all stages of pregnancy showed considerable variation. During the first month of pregnancy the vitamin C content of the milk varied from 14.34 mg. per liter for cow no. 18 to 22.84 mg. for cow no. 13. Likewise, during the fourth month of pregnancy the vitamin C content of the milk varied from 9.84 mg. per liter for cow no. 12 to 19.34 mg. per liter for cow no. 18.

SUMMARY

Milk produced by Guernsey and Holstein cows at various stages of pregnancy was assayed for its ascorbic acid content.

The highest average value was found in the milk of the Guernsey cows during the first month of pregnancy, namely, 22.47 mg. per liter. A gradual but not consistent decrease in the average vitamin C content was observed in the milk from Guernsey cows as pregnancy advanced.

The data obtained for the Holstein cows were not in agreement with those yielded by the Guernsey cows. The highest ascorbic acid content of the milk from this group of cows was found in the sixth month of pregnancy. The increase in vitamin C content of milk from the Holstein cows during the fifth, sixth and seventh months of pregnancy is unexplained.

The data obtained in this study indicate that the vitamin C content of milk from Guernsey and Holstein cows tends to decrease with the advance of pregnancy, but at all stages of pregnancy different cows show considerable variation.

ACKNOWLEDGMENT

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ENERGY AND GASEOUS METABOLISM OF THE HEN AS AFFECTED BY TEMPERATURE

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SEVEN FIGURES

(Received for publication February 17, 1941)

INTRODUCTION

Constancy of body temperature of a homoiotherm is maintained even under extreme variation in environmental conditions. To maintain this constant temperature there must always be a balance between the heat production within the system and loss of heat from the system. The heat produced is supplied by the oxidation of feedstuffs or of material stored within the body. Heat is lost by radiation, convection, and as latent heat of vaporization of water. It is apparent then that environment may affect the life processes. A measurement of the metabolism is a measurement of the activity of these life processes and by studying the course of metabolism under different conditions of environment some information may be gained as to the effect of environmental conditions on the life processes.

Several investigators have studied the effect of temperature on the gaseous metabolism of the hen by use of the open circuit Haldane method. Mitchell and Haines ('27) made observations on the carbon dioxide elimination of hens at nine different environmental temperatures with 5-degree intervals between 45° and 85° F., inclusive. They concluded that there was a critical temperature (the temperature at which the metabolism is at a minimum) at 62° F. Our analysis of their

published results gives a very different temperature for the minimum metabolism. Their data were reanalyzed to obtain the carbon dioxide elimination per hour per gram of live weight of the hen and a mean value calculated for each observed temperature. These values were plotted (fig. 1) and a curve was drawn through them. This curve shows that the metabolism, as indicated by elimination of carbon dioxide, is at a minimum between 75° and 80° F.

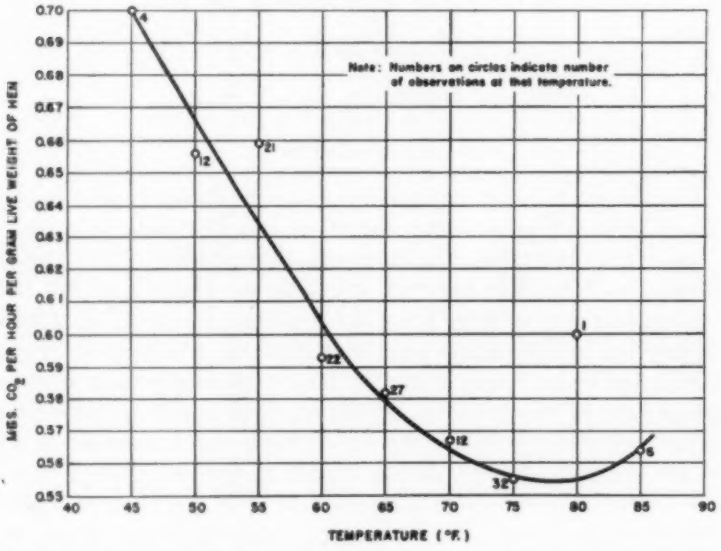

Fig. 1 Carbon dioxide elimination of hens at various temperatures. Source: Data of Mitchell, H. H., and W. T. Haines.

Terroine and Trautman ('27), using the open circuit Haldane method, studied the effect of temperature on the gaseous metabolism of several species of homoiotherms, among them the hen. They used birds whose weights ranged between 854 and 1,075 gm. Neither the breed nor the age of the birds is given although the article states that all animals studied were adults, but from the weight one must conclude that the hens, if adult, were undersized. The data given were for fourteen

experiments at ten different temperatures between 15° and 99° F. The results show a minimum metabolism at 78° to 80° F. The metabolic rate increased with either an increase or decrease in temperature. At 99° F. and also at 26° F. the rate was nearly twice that at 80°, while at 16° F. it was two and one-half times that at 80° where the minimum was found.

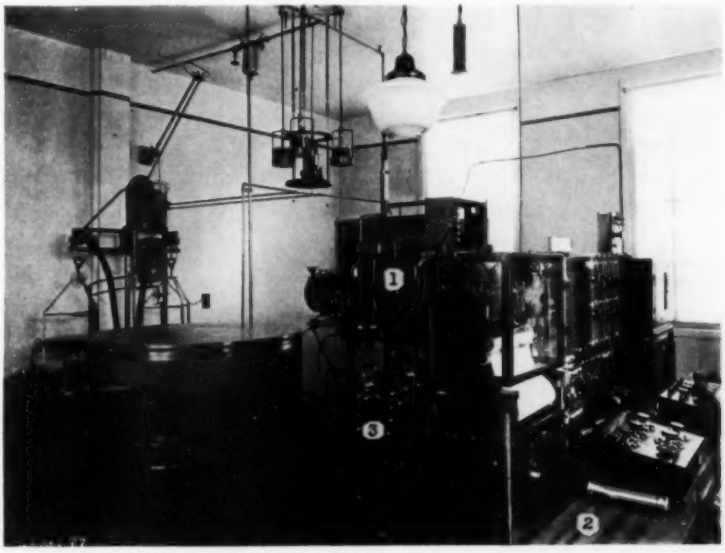

Fig. 2 Respiration calorimeters and accessory apparatus. 1, calorimeter used for this research; 2, board with instruments for measurement and control; 3, train for absorption of water and carbon dioxide.

PROCEDURE

The results herein reported on the effect of temperature on the metabolism of the hen were obtained by use of one of the respiration calorimeters in the Calorimetry Section of the Bureau of Animal Industry, Beltsville Research Center, Beltsville, Maryland, between April, 1938 and April, 1939. This calorimeter (fig. 2) is an instrument of precision. The construction and technique of operation are in every way identical

with the one described briefly by Barott, Byerly and Pringle ('36), and in greater detail by Barott ('37). The capacity of the system is approximately 130 l. and the flow of air to and from the chamber approximately 90 l. per minute.

In this series of experiments Rhode Island Red hens from 10 to 13 months of age were used. These hens were kept in a colony house except during an observational period and were provided with the regular laying diet. The following routine was used: A hen was brought from the house to the laboratory at daybreak. Therefore, having had no feed since the previous

Fig. 3 Hen on perch inside cage, indicating the type of confinement during the experimental period. The semi-circular "cut-out" in front side near top is made so the wings may remain outside cage during experiments at the higher temperatures.

night, the alimentary tract was comparatively empty. The hen was weighed and placed on a wooden perch with a specially constructed copper wire cage attached (fig. 3). This cage was of such size as to confine the bird closely so that movement was kept at a minimum. The cage containing the hen was then placed in the calorimeter. A dish containing liquid petrolatum was placed back of the perch in such a position that the droppings would fall into the dish. The oil prevented the addition of water to the chamber by evaporation from the feces. The calorimeter was then closed and

sealed. The conditions that were to prevail relative to temperature and humidity were established so that the experiment could be started at 9 A.M. The total energy and gaseous metabolism were determined for one 3-hour period from 9 A.M. to noon. Thereafter, the oxygen was obtained for each 2-hour period and the water, carbon dioxide, and heat for each 4-hour period. At 8 A.M. the next day the calorimeter was opened and the hen removed and weighed. The weight of the excrement was obtained by taking the difference in weight of the dish of oil and contents before and after the experimental period.

The initial weight of the hen minus the weight of the droppings was used in computing the first value for metabolic rate. The final weight was used in computing the last value. To compute the intermediate values the difference between these two weights was prorated lineally with time.

During the experimental period the air temperature within the calorimeter was kept practically constant at a predetermined value, the relative humidity was kept between 50 and 60%, the oxygen content at 21%, and the carbon dioxide below 1%.

The experiments covered a range of temperature from 50° to 95° F. Observations were made at nineteen different temperatures within this range. One-day experiments were conducted using a different hen for each experiment. The metabolic rate for each temperature represents a mean of the results of several 1-day experiments. The number at each temperature is shown in figure 5.

Energy and gaseous metabolism

The data obtained for each hen were analyzed and the metabolism per hour per gram of live weight was calculated. These final values were plotted and a curve drawn through the plotted points in such a manner that there was an equal distribution of points on each side. Figure 4 [1] shows a typical

[1] It will be noted that some points are plotted at times differing from the mean of the 2-hour or 4-hour periods, or that a point is missing. This is because the data were incomplete for that period. In some cases it was possible to combine two consecutive periods and obtain a value.

example. These data were determined at a temperature of 84.8 $\pm$ 0.4° F. and are the results obtained with five different hens. The curve was extrapolated to 8 A.M. From previous data (Barott et al., '38), it is known that the maximum rate of metabolism occurs at 8 A.M.; consequently, this extrapolation can be made with small error.

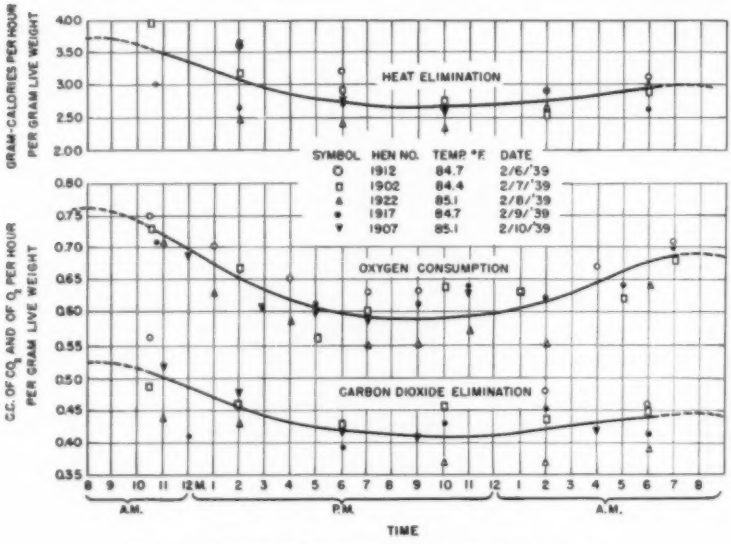

Fig. 4 Energy and gaseous metabolism of the hens.

These curves show the typical diurnal rhythm in the metabolic rate of the fowl, with a high value at 8 A.M., the rate declining until it reaches a minimum at 8 P.M., thereafter rising again until 8 A.M. The value at 8 A.M. of the second day is approximately 10% lower than that of the first day due to the fact that the birds were without feed during their stay in the calorimeter. The decrease in metabolic rate for these hens during the first day was less than 50% of that for immature birds 9 to 10 weeks of age, as previously determined (Barott et al., '38).

Charts similar to figure 4 were constructed for the results obtained at each temperature studied. These charts show the

metabolic rate during the experimental period as measured by heat and carbon dioxide elimination and oxygen consumption. By integration of the area under the curves of these charts the metabolism may be obtained for any time interval during the day.

By such integration the values were found for carbon dioxide and heat elimination and for oxygen consumption for the first 12 hours at each temperature. The mean values per hour were calculated and plotted in figure 5 and curves drawn

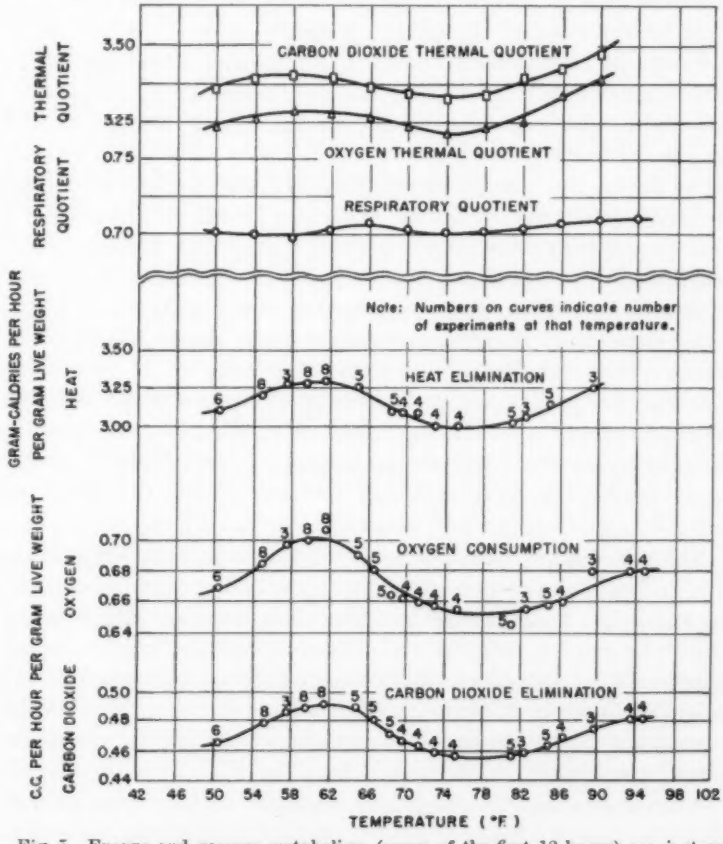

Fig. 5 Energy and gaseous metabolism (mean of the first 12 hours) respiratory quotient and carbon dioxide and oxygen thermal quotients.

through the plotted points. These values show the effect of temperature over the range from 50° to 95° F. on the metabolic rate, as indicated by these three quantities. The three curves are similar in form, each having two points of flexure, a minimum value occurring at approximately 78° F. and a maximum at approximately 61° F. The rate at 61° F. is approximately 8% higher than that at 78° F. As the temperature is increased above 78° F. the metabolic rate increases to the highest temperature (95° F.) studied. At 95° F. several of the hens died and therefore the authors did not attempt to conduct experiments at higher temperatures.

From the results obtained by Terroine and Trautman ('27) and Mitchell and Haines ('27), it was expected that the metabolic rate would increase steadily as the temperature was decreased below 60° F. Instead of doing so, the rate became lower when the temperature of 61° F. was passed and continued to decrease to 50° F. with the trend still downward and the rate approaching that at 78° F. The authors have conducted no experiments below 50° F. to date. It seems probable that the curve representing the metabolic rate has another point of flexure not many degrees below 50° F., after which the metabolic rate increases once more. If this were not true the fowl would be unable to compensate for the loss of heat due to low external temperatures and would perish. More work in the lower temperatures must be done in order to determine the metabolism of the fowl in this temperature zone.

The respiratory quotient and the oxygen and carbon dioxide thermal quotients were computed from the values for oxygen consumption and heat and carbon dioxide elimination taken from the curves in figure 5. The respiratory quotient thus obtained (for the mean 12-hour period from 8 A.M. to 8 P.M.) is approximately 0.702 $\pm$ 0.002 between 50° and 80° F. The respiratory quotient becomes greater at the higher temperatures, reaching a value of 0.710 at 94° F.

The oxygen and carbon dioxide thermal quotients are respectively 3.25 $\pm$ 0.05 and 3.37 $\pm$ 0.05 in the temperature range

from 50° to 80° F. The thermal quotients became greater for temperatures above 75° F. as in the case of the respiratory quotient, and at 90° F. are 3.39 and 3.45, respectively.

The respiratory quotient and oxygen and carbon dioxide thermal quotients indicate that fat constituted nearly all of the material being metabolized by the hens, which probably is true since they had no feed for approximately 20 hours before 2 P.M., the mid-point of the 8 A.M. to 8 P.M. period for which the metabolic rate was determined.

Water metabolism

The discussion thus far has concerned energy and gaseous metabolism. The elimination of respiratory water was also determined in this research. The water varies to a great extent with the environment of the fowl.

Birds have no sweat glands in the skin. Therefore, when it is necessary to eliminate heat in order to control the temperature of the body, they do so by the great quantity of air they are able to inhale and exhale with each breath. This air passes through the lungs, which are comparatively small, compact, and inelastic, and into nine air sacs, which are distributed throughout much of the body and fit closely around the viscera and muscles, thus affording a method of eliminating heat by evaporation of large quantities of water from the tissue surfaces.

It was noted in our work that at the higher temperatures panting occurred and that the higher the temperature, the more pronounced was the panting. This fact led us to analyze the course of water elimination over the range of temperature studied. Figure 6 shows the rate of elimination of respiratory water, derived by computations of water elimination from our observed data. Between the temperatures of 65° F. and 75° F. the rate of elimination remains nearly constant. At temperatures below 65° F. the rate decreases at first slowly and then more rapidly as conservation of heat becomes necessary because of lower temperatures.

At temperatures higher than 75° F. the rate of elimination begins to increase and after a temperature of 80° F. is passed the amount of water thus eliminated increases very rapidly, so much so that at 90° F. over three times as much water is being eliminated from the respiratory system as was eliminated at 80° F. Above 90° F. the rate still increases slightly to 95° F. but it is apparent that the hen is approaching the point at which she can no longer increase her capacity for

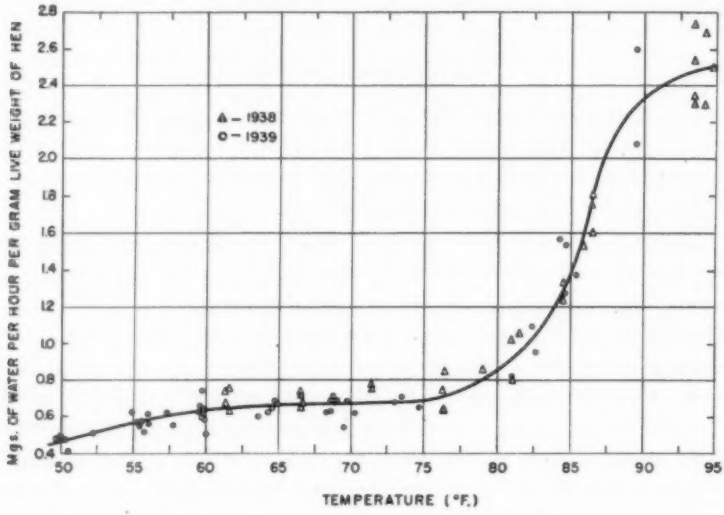

Fig. 6 Respiratory water eliminated by hens at different temperatures and at 50% relative humidity.

cooling her body by breathing, and the rate of increase in water elimination, as shown by the curve, seems to be approaching zero.

The fact that several of the hens died at a temperature of 95° F. would seem to be strong evidence that they could not longer eliminate sufficient heat, because they could no longer increase their "ventilation", and so died of heat prostration.

To prove conclusively that this was true, a series of observations were conducted with the same or similar hens at tempera-

tures between 90° F. and 100° F., and at relative humidities between 30 and 80%. It was found that hens at a given temperature would survive at the lower humidities but would die when the humidity was increased. At 90° F. all hens lived for 14 hours (the duration of the test) at all humidities below 75%. At 95° F. hens survived at humidities below 60% but perished at higher humidities; while at 100° F. they survived only at humidities of 30% or lower.

Another interesting phenomenon was noted. Some hens died at comparatively high temperatures while others subjected to the same treatment survived. Upon examination of the dead hens, in each case it was found either that the hen was very fat, the liver or internal organs were enlarged, or there was a large cluster of egg yolks in the body cavity which impeded the dilation of the air sacs. A hen without excessive fat and in such condition that its air sacs could be utilized to capacity survived with no noticeable ill effects.

The great increase in water elimination in the temperature region between 80° and 95° F. and the fall in metabolic rate at temperatures between 60° and 50° F., induced the authors to analyze the sensible and latent heat elimination over the temperature range studied.

All the data were tabulated for each observed temperature and a mean of the values for both latent heat and sensible heat at that temperature plotted in figure 7. A graph which shows the relative values for the sensible and latent heat elimination was drawn through the plotted points.

A third graph was added which shows the total heat elimination. This third graph is similar in form to that in figure 5. However, the values are somewhat lower for they represent more nearly the minimum for the 24-hour period, whereas the curve (fig. 5) represents the mean for the period from 8 A.M. to 8 P.M.

It may be noted (fig. 7) that both latent and sensible heat remain practically constant over the temperature range between 65° F. and 80° F. Between 65° and 60° F. the sensible heat tends to increase and the latent heat tends to decrease, but

the sensible heat increases much more rapidly than the latent heat decreases so that the total heat elimination is increased to compensate for the lower environmental temperatures.

One would expect the total heat elimination to continue to increase as the temperatures were lowered below 60° F. but, surprisingly, just the opposite happened. The latent heat continued to decrease, as would be expected, but the sensible heat also dropped rapidly between the temperatures of 60° and 55° F. At temperatures between 55° and 50° F. the curve

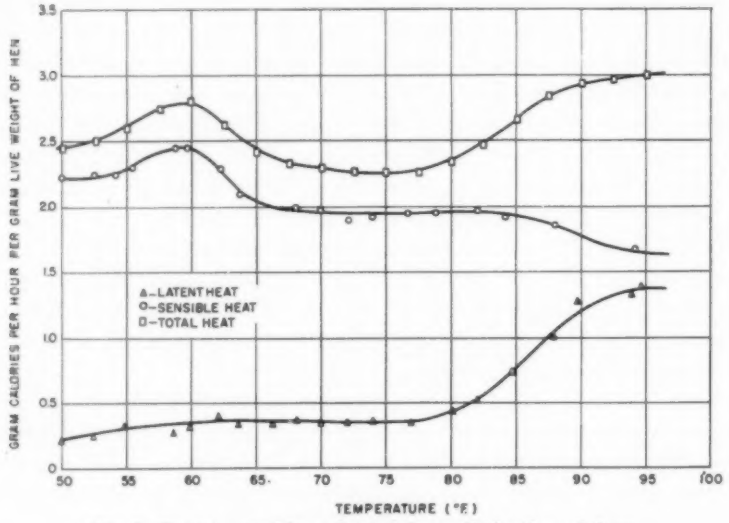

Fig. 7 Latent, sensible and total heat elimination of hens.

representing sensible heat appears to be approaching a third point of flexure. It appears probable that at temperatures lower than 50°F. the latent heat would continue to decrease and that the sensible heat would again begin to increase. However this is only a conjecture and the proof of this as well as the reasons for the drop in metabolic rate at temperatures below 60° F. must await further investigations.

The data show that in the region between 65° and 75° F. both the latent and sensible heat remain practically constant

under the experimental conditions. When the temperature is increased above 80° F. the latent heat begins to increase rapidly, indicating a large increase in water elimination in the respired air. Above 85° F. the latent heat increases rapidly to 95° F. while the sensible heat decreases in an effort to maintain the body temperature. The data indicate that at 95° F. and 60% humidity the fowl is approaching a state where the sensible heat can no longer be appreciably decreased or the latent heat increased.

SUMMARY AND CONCLUSIONS

The energy and gaseous metabolism of Rhode Island Red hens between 10 and 13 months of age were determined by use of one of the respiration calorimeters in the calorimetry section of the Bureau of Animal Industry, Beltsville Research Center, Beltsville, Maryland. The instrument is similar to the one described briefly by Barott ('37).

Results were obtained at nineteen environmental temperatures in the range from 50° to 95° F. Conditions other than temperature were relative humidity 60%, oxygen content 21%, and carbon dioxide content less than 1%.

The oxygen consumption was measured for each 2-hour period, and the heat, water, and carbon dioxide elimination for each 4-hour period. From the above data the respiratory quotient and the oxygen and carbon dioxide thermal quotients were computed.

The data obtained during each 24-hour period show the typical diurnal rhythm in the metabolism of the hen with a maximum value in the morning and a minimum in the evening.

The results clearly define the metabolic rate of the Rhode Island Red hen for the temperature range studied. A point of flexure occurs at 78°F. This is the temperature of minimum metabolism of the hen. The maximum metabolism occurs at 61° F., at which temperature there is also a point of flexure. The phenomenon of a decrease in metabolic rate between 60° and 50° F. should be investigated to ascertain the reason for

the decrease instead of the expected increase in metabolic rate with decrease in temperature.

The respiratory quotient was 0.702 ± 0.002. The oxygen and carbon dioxide thermal quotients were 3.25 ± 0.05 and 3.37 ± 0.05, respectively, indicating a fat metabolism. This is consistent with the fact that the data were obtained approximately 20 hours after feeding.

The rate of elimination of respiratory water was approximately constant between 65° and 75° F.

The rate decreased considerably between 60° and 50° F. but the most striking change occurred between 80° and 90° F. The rate at 90° F. was three times that at 80° F. This was due to the large amount of air inhaled and exhaled for cooling at the high temperature.

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THE EFFECTS OF VARIOUS VITAMIN SUPPLEMENTS AND OF WHOLE YEAST ON THE DIGESTION AND ABSORPTION OF THE CARBOHYDRATE OF A COMPLETE DIET[1,2]

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ONE FIGURE

(Received for publication April 10, 1941)

There are many reports in the literature linking the vitamin B-complex and the gastrointestinal tract, and in many of these studies multiple deficiencies probably existed. There is a tendency to regard thiamine as specially important in the maintenance of the normal function of the gastrointestinal tract, but experiments clearly showing such a relationship are rare. Few workers have interested themselves in the effects of supplementing what is currently considered an adequate diet with vitamins in excess of the minimum requirements for growth. There have been few studies on the adult animal, whose requirements may conceivably differ considerably from those of the young and rapidly growing one. Furthermore, most earlier vitamin work necessarily was concerned with the minimal requirements, whether for growth or maintenance, and the measure of such requirement was based largely on the rate of change or the maintenance of body weight.

[1] The data in this paper are taken from a thesis presented by R. A. Russell to the Division of Graduate Studies, University of Rochester, in partial fulfillment of the requirements for the degree Master of Science, 1940.

[2] This investigation was supported by a grant from Standard Brands, Inc.

The object of this investigation was to determine the effect, if any, of large amounts of the B-complex on the function of the normal gastrointestinal tract of the adult animal as contrasted with the growth of young animals. To this end, amounts of the B-complex well in excess of the accepted dietary requirements were fed to dogs maintained on a basal ration known to be adequate for growth, maintenance, and reproduction. The effects of such supplements on the digestion and absorption of carbohydrates and gastrointestinal motility were studied.

METHOD

Maydl jejunostomies were established 50 to 75 cm. from the ligament of Treitz in five healthy adult male dogs. A catheter could be inserted into the lumen of the gut whenever desired and its contents withdrawn, whereas normally they passed the stoma without leakage. These animals were maintained in excellent condition on a basal diet [3] which is known to fulfill the ordinary dietary requirements of both the dog and the white rat. Various supplements were fed with this diet from time to time, experimental and basal periods being alternated so that any progressive changes in gastrointestinal function could be detected.

Only after 6 to 8 days on a particular diet were the animals used for collection of intestinal contents. About 20 hours after their last feeding, the animals were placed in stalls and a no. 24 French soft-rubber catheter with several holes in it inserted into the jejunostomy. After 30 minutes of aspiration at −12 cm. of water pressure to remove residual material, the animals were fed a standard test meal consisting of 100 gm. of finely-ground basal ration moistened with about 50 ml. of water. Thus, with the exceptions noted, the performance of the upper part of the digestive tract was always tested on the basal ration regardless of what supplements the animal might be ingesting with his regular diet. In some experiments the supplements were fed with the test meal to determine whether

[3] Purina Dog Chow.

there was local as well as systemic conditioning of the alimentary tract. The chyme was collected at 15-minute intervals, the volume and weight of each sample recorded, and pH determinations made frequently with a glass electrode. Each sample as collected was divided into two equal parts: one was boiled immediately to stop digestion and the other was incubated under toluene at 38°C. until enzyme hydrolysis was complete (48 hours). All samples for each experiment were thus divided and pooled in two flasks, the reducing substance in each was extracted with water, proteins were precipitated with $Zn(OH)_2$, and the total reducing substance determined by the Shaffer-Somogyi ('33) method.

The "final emptying time" was fixed by several criteria: (1) recovery of 20 cc. or less of intestinal contents in a half-hour period, (2) absence, or at most traces, of solid food particles, (3) a pH typical of pre-feeding contents (6.0–6.5), and (4) a negative Benedict's test for reducing sugar. The collection was continued for an hour or more after the final emptying time had been determined in order to make certain that the intestine was empty.

From the analytical results and the time required for the final appearance of food, the average rates of carbohydrate digestion and absorption were calculated. The test meal contained 43.4% total carbohydrate, including 10.1% reducing substance. In calculating the rates of digestion it was assumed that the 10.1% of reducing substance was absorbed first and required no digestion. This amount subtracted from the sum of absorbed and recovered reducing substance gave the amount digested. This accounts for the apparently anomalous situation that the animal absorbed more than he digested (table 1).

The *t* test as described by Fisher ('34) for small samples was used in calculating the significance of differences observed between the rates of digestion and absorption on the several experimental diets, and the rates on the unsupplemented basal diet.

RESULTS

Table 1 summarizes the results obtained with five dogs.

TABLE 1

Carbohydrate digestion and absorption as affected by fresh yeast.

DOG NO.	CARBOHYDRATE ABSORBED PER HOUR			CARBOHYDRATE DIGESTED PER HOUR		
	Basal ration	Basal ration plus yeast	Change	Basal ration	Basal ration plus yeast	Change
	gm.	gm.	%	gm.	gm.	%
1	5.75 (14)[1]	6.87 (7)	+19	5.09	5.94	+16
2	7.03 (4)	7.81 (4)	+11	5.70	6.03	+ 6
3	6.98 (3)	7.06 (3)	+ 1	5.88	5.72	— 3
4	6.78 (5)	7.67 (3).	+13	5.58	6.07	+ 9
5	10.24 (4)	11.46 (2)	+12	8.22	8.99	+ 9

[1] Number of experiments averaged in parentheses.

The first supplement used in this study was fresh commercial foil-wrapped yeast, which was fed at the level of two cakes (about 26 gm.) per day. It was found that feeding yeast with the test meal had a pronounced effect on gastrointestinal motility, producing larger initial volumes and a shorter final emptying time (fig. 1). Comparison with the

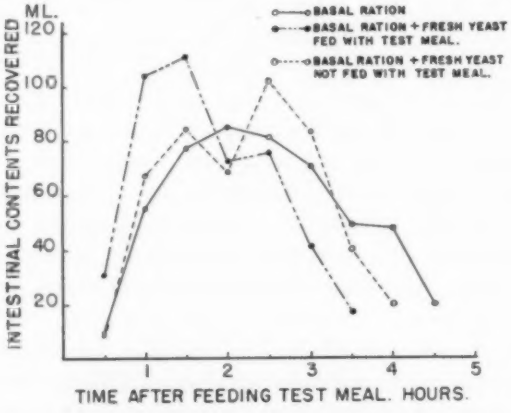

Fig. 1 Test meal recoveries from jejunostomy in dog 1. Basal ration: 22 experiments. Basal ration and fresh yeast—yeast fed with test meal: 7 experiments. Basal ration and fresh yeast—yeast not fed with test meal: 7 experiments.

curve for a series of experiments where yeast was not fed with the test meal, although the animal was on a yeast regime, reveals that this effect is largely local in nature, owing to the presence of live yeast in the gastrointestinal tract. The use of two cakes of fresh yeast with the test meal causes fermentation and makes carbohydrate analyses meaningless. It was found, however, that even without the presence of live yeast in the test meal, the rates of digestion and absorption were increased in four of the five dogs (table 1), although the effects on motility were greatly reduced. This suggests that fresh yeast acts in at least two ways on the gastrointestinal tract, namely, its actual presence has a pronounced effect on motility, and its regular feeding prior to experiment conditions the alimentary tract so that digestion and absorption are more rapid.

With the observation of these effects experiments were begun to determine if the crystalline vitamins [4] then available were the causative agents. Supplements containing the yeast equivalents of crystalline thiamine (1.3 mg.), riboflavin (0.6 mg.), nicotinic acid (1.0 mg.) and pyridoxine (0.4 mg.), together with highly concentrated preparations of vitamins A and D, were administered daily with the basal ration instead of the yeast. It was found that such supplements whether given with the test meal or not had no effect either on gastrointestinal motility or on the rates of digestion and absorption. Five and ten times these amounts were similarly without effect, showing conclusively that this combination of vitamins was not responsible for the yeast effects. This indicates also that feeding excessive amounts of these factors, over and above the ordinary dietary requirements, has no effect on the gastrointestinal functions as studied in this investigation.

The baker's yeast was then dried and tested on the same animals at an intake equivalent to two cakes of fresh yeast per day. It was found to produce about the same increase in the rates of digestion and absorption, without influencing motility. The 50% alcohol-soluble fraction of dried yeast con-

[4] Kindly supplied by Merck and Company, Inc.

tained most of the active principles. Even when fed with the test meal, neither the dried yeast nor the extract gave the effects on volumes that were observed when fresh yeast was fed.

Shortly before the conclusion of this work crystalline d-l-calcium pantothenate became available and 2 mg. per day were added to a vitamin supplement of thiamine, riboflavin, nicotinic acid, and pyridoxine, which previously had been without effect on the gastrointestinal tract. The addition of calcium pantothenate was accompanied by a marked increase in

TABLE 2

Summary: dog 1. Carbohydrate digestion and absorption on several diets.

DIET		CARBOHYDRATE ABSORBED PER HOUR				CARBOHYDRATE DIGESTED PER HOUR			
No.	Description	Grams	% above basal	t[1]	P[2]	Grams	% above basal	t[1]	P[2]
1	Unsupplemented basal ration (14)[3]	5.75				5.09			
2	No. 1 plus fresh yeast (7)	6.87	19	2.34	0.03–0.04	5.94	16	2.20	0.04–0.05
3	No. 1 plus dried yeast (3)	7.04	22	3.12	less than 0.01	5.82	14	2.16	0.04–0.05
4	No. 1 plus yeast extract (3)	6.91	20	2.79	0.01–0.02	6.27	23	2.92	0.01–0.02
5	No. 1 plus thiamine, riboflavin, pyridoxine & nicotinic acid (5)	5.84	1	0.23	0.8–0.9	5.01	−2	0.25	0.8–0.9
6	No. 5 plus d-l-calcium pantothenate (3)	8.67	51	6.64	less than 0.01	6.95	37	5.24	less than 0.01

[1] When compared with unsupplemented basal ration.
[2] Probability that difference occurred by chance.
[3] Number of experiments averaged in parentheses.

the rates of digestion (51%) and absorption (37%) of carbohydrate in the three experiments in which it was used. These experiments strongly suggest that pantothenic acid is responsible for at least a part of the efficacy of whole yeast.

In table 2 is given a summary of the rates of digestion and absorption of dog 1 on the several experimental diets de-

scribed, together with t values and the probabilities of a chance deviation of the results on supplemented diets from the control diet.

On all of the diets used a consistent inverse relationship between volume and pH was observed. Small samples such as were recovered at the beginning and end of alimentation showed a pH range of 6.0–6.5 or higher, while the larger samples recovered at the height of the process ranged from 4.5 to 6.0. Rarely did the pH of the intestinal contents exceed 7.0.

DISCUSSION

The evidence shows that digestion and absorption of carbohydrate may be hastened by adding whole B-complex in the form of yeast to a diet currently considered adequate in all respects. It is generally conceded that adding an excess of certain individual members of the B-complex to a complete diet has no effect. The present experiments on the use of thiamine, riboflavin, nicotinic acid, and pyridoxine confirm this idea with respect to the gastrointestinal tract. However, the numerous experiments with whole yeast and a few experiments with pantothenic acid suggest that what currently passes for a complete diet may not, in fact, be adequate for optimal function in all respects.

It is known that yeast extracts excite the secretory cells of the small intestine and stomach (Boldyreff, '31; Bourns, Nasset and Hettig, '36; Williams, Cox and Nash, '40) and this fact may account in part for the accelerated digestion and absorption noted in the present work.

SUMMARY

The effects of various vitamin supplements and of whole yeast on digestion and absorption were studied in dogs with jejunostomies.

It was found that fresh yeast has a distinctly stimulatory effect on gastrointestinal motility, which is largely local in nature, and probably due to the presence of live yeast in the gastrointestinal tract.

The increased motility is often but not always accompanied by increased rates of digestion and absorption of carbohydrate.

Dried yeast and a 50% alcohol-soluble extract of this material have no effect on motility but increase the rates of digestion and absorption by about 20%.

Supplementing an already adequate basal diet with additional amounts of thiamine, riboflavin, nicotinic acid, and pyridoxine has no effect on gastrointestinal tract measurable by the technic employed.

Evidence is presented indicating that crystalline pantothenic acid may be responsible for at least part of the yeast effect.

Mr. John Lambooy was kind enough to prepare the 50% alcohol-soluble yeast extract.

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FURTHER OBSERVATIONS ON RIBOFLAVIN AS A FOOD FACTOR IN ECONOMY OF FOOD UTILIZATION [1]

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ONE FIGURE

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In a recent communication (Sure and Dichek, '41) it was demonstrated that riboflavin produces a pronounced effect on economy of food utilization for synthesis of body tissues and that the increases in body gains were derived mainly from fats and to a lesser extent from proteins. The data submitted represented results of studies on thirty-three pairs of animals on two types of diets, the control animals having been restricted to the same amount of food consumed by the litter mate riboflavin deficient rats. Nothing was mentioned in the recent paper concerning the pathological symptoms and the plane of nutrition of the riboflavin deficient animals during various stages of this avitaminosis, and it was not clear whether, as in thiamine deficiency, marked anorexia complicated the final collapse from riboflavin deficiency.

In this study evidence is submitted to the effect that, unlike in thiamine deficiency, the final collapse in riboflavin deficiency may be associated with either moderate reduction, no reduction, or on the contrary even greater food intake in final stages compared with early stages of riboflavin deficiency. In fact, it would seem that some riboflavin deficient animals in the

[1] Research paper no. 675, Journal Series, University of Arkansas. This paper is XXII in the series of AVITAMINOSIS. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

later stages of deficiency behave very much like diabetics, exhibiting abnormal hunger, thus craving compensation for inadequate utilization of foods.

This study was carried out with ten pairs of male rats, according to technique previously described (Sure and Dichek, '41). Five pairs were given our diet 13 of the following percentage composition: Casein,[2] 18; salts,[3] 4; butterfat, 10; cod liver oil, 2; and dextrin, 66. The dextrin carried an 80% alcoholic extract of 25 gm. rice polishings in a 100 gm. ration, as a source of all the components of the vitamin B complex with the exception of riboflavin. Each animal also received daily 10 μg. thiamine and 10 μg. pyridoxine, and the control animals received in addition 20 μg. riboflavin daily.

Another five pairs of male rats were given a diet with the following percentage composition: Casein (vitamin-free),[4] 18; agar-agar, 2; nicotinic acid, 0.05; salts,[5] 4; butterfat, 10; and dextrin, 65.95. This ration was supplemented with daily amounts to each animal of 20 μg. thiamine, 20 μg. pyridoxine, 6 mg. choline chloride, 100 μg. calcium pantothenate, and 150 mg. of rice polish factor II (Supplee, Bender and Kahlenberg, '40). The control animals also received 20 μg. riboflavin daily. As a source of vitamins A and D, 2 drops of halibut liver oil was given once weekly to each animal.

The results of this investigation are submitted in table 1 and in figure 1.

In table 1 additional evidence is submitted showing that riboflavin produces a marked influence in economy of food utilization. Whereas, during an average experimental period of 125 days, the average gain in body weight of the riboflavin deficient animals was 6.1 gm. per rat, during the same period of time on the same amounts of the same ration the litter mate controls, which had received 20 μg. riboflavin daily, gained 61.3 gm. per animal. We have since determined that

[2] Thoroughly washed with acidulated water and dilute ethyl alcohol.

[3] Phillips, P. H., and E. B. Hart. The effect of organic dietary constituents on fluorine toxicosis in the rat. J. Biol. Chem., vol. 109, p. 658, 1935.

[4] Supplied by the Borden Company, New York, under the trade name "Labco."

[5] See footnote 3.

optimum results are obtained in riboflavin studies of economy of food utilization when animals are taken at initial weight of 55 to 70 gm. If the recommendations of Day, Darby and Cosgrove ('38) are followed, in starting with animals weighing

TABLE 1

Riboflavin as a food factor in economy of food utilization

PAIR NUMBER	RATION NUMBER	PERIOD OF EXPERIMENTATION	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT	AVERAGE DAILY FOOD INTAKE DURING FIRST 10 DAYS OF EXPERIMENTAL PERIOD	AVERAGE DAILY FOOD INTAKE DURING LAST 10 DAYS OF EXPERIMENTAL PERIOD
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1–P[1]	13	115	72	67	—5	6.36	8.65
RC			80	161	+81		
2–P[1]	13	107	72	60	—12	6.47	6.09
RC			80	100	+20		
3–P[1]	13	152	72	70	—2	7.40	5.38
RC			71	157	+86		
4–P[1]	13	80	72	49	—23	4.98	4.08
RC			84	82	—2		
5–P	13	161	61	74	+13	5.99	4.90
RC			61	130	+69		
6–P[1]	14	148	70	88	+18	7.37	5.23
RC			65	140	+75		
7–P[1]	14	106	77	58	—19	6.06	4.48
RC			70	92	+22		
8–P	14	115	64	130	+66	8.07	7.79
RC			63	171	+108		
9–P	14	115	70	100	+30	7.40	4.90
RC			64	116	+52		
10–P[1]	14	150	59	54	—5	6.84	6.93
RC			60	162	+102		

[1] P = pathological or riboflavin deficient animal; RC = restricted control. In every pair except 5, 8 and 9 the period of experimentation was determined by the death of animal P.

from 30 to 45 gm., in order to insure the production of cataracts, such small animals take many weeks to adjust themselves to the new experimental rations, during which periods they consume very small amounts of food, so that the results in economy of food utilization are not as clear-cut as when

larger animals are selected at the beginning of the experiments. For this reason, the data submitted in this paper show riboflavin as producing much greater economy in food utilization than the findings reported in our recent paper (Sure and Dichek, '41).

We have noted that during the later stages of deficiency, even near the point of collapse, the riboflavin deficient animals are ingesting on the average very nearly the same amounts of food as they did during the first 10 days on the experiments when the daily food intake is generally at its optimum. Therefore, when an average of food consumption is taken for the first 10 days and the last 10 days of the experiments of animals which ultimately died from riboflavin deficiency, it is possible to determine to what extent, if any, anorexia has been a complicating factor in the terminal stages of this deficiency disease.

Of the ten pairs represented in this study, seven pathological animals died and the changes in the average daily food intake during the last 10 days compared with the first 10 days of the experimental periods were as follows: +36%, −6%, −27%, −18%, −29%, −26%, and +1%.

The average daily food intake during the last 10 days compared with that for the first 10 days of the experimental period for seven riboflavin deficient animals that died, results of which have recently been reported (Sure and Dichek, '41) are as follows: +12%, −21%, −5%, +4%, −6%, and +45%. Averaging all these figures for thirteen animals that succumbed from riboflavin avitaminosis we obtain a reduction of only 3%. The pathological animals of pairs 1 and 10 were actually eating more food daily preceding their total collapse than at any time during the entire experimental periods. The riboflavin deficient rat in pair no. 10 consumed 7 gm. during the 24-hour period preceding his death and was observed eating just 1 hour before he died. This is an entirely different picture from that encountered in thiamine deficiency, which produces in the last stages an inanition so pronounced as to approximate starvation (Karr, '20; Cowgill, '21; Cowgill and associates, '25 and Sure, '28). We also frequently en-

counter pronounced anorexia in terminal stages of vitamin A deficiency. This certainly does not take place in riboflavin deficiency. Since the anorexia referred to in our recent communication (Sure and Dichek, '41) was cured with the daily administration of 10 μg. pyridoxine to each animal, it is most probable that we were dealing with a pyridoxine deficiency rather than a lack of riboflavin.

The pathological symptoms noted in our riboflavin deficient animals are as follows: Alopecia, frequently accompanied by

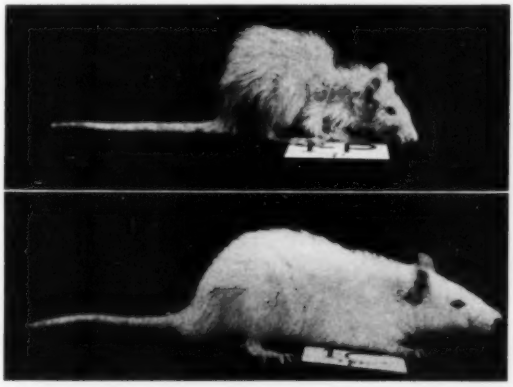

Fig. 1 Rats of pair no. 10, 1-P being riboflavin deficient and 1-C being normal. Both received the same amount of food of the same ration, but 1-C was given in addition 20 μg. riboflavin daily. Photographs taken 1 day before the 1-P animal died. Weight of 1-P, 54 gm. Weight of 1-C, 162 gm. The 1-P was eating about 7 gm. daily during the last few days of his life but was unable to utilize his food which resulted in his death. On the same daily food intake 1-C, receiving riboflavin, was still growing. Note the rough coat, alopecia, conjunctivitis and senility of the 1-P animal.

dermatitis and ulcerations in the denuded areas, rough hair, conjunctivitis, and keratitis, occasionally associated with a discharge of a granular exudate, and in the terminal stages, muscular incoordination evidenced by the animals walking on their tip-toes and their inability to balance properly on the hind legs. Premature senility is apparent in all cases of advanced stages of riboflavin deficiency. A typical illustration is submitted in figure 1.

The question arises as to what causes animals to collapse in the terminal stages of riboflavin deficiency when they are at the optimum plane of nutrition commensurate with the character of diet at their disposal. The findings of (1) Ochoa and Rossiter ('39) that there is a decrease in the total riboflavin-adenine dinucleotide content of the heart and liver, (2) Axelrod, Sober and Elvehjem ('40) that there is a decrease in the amino acid oxidase in liver and kidney in riboflavin avitaminosis, and (3) particularly the demonstration of Warburg and Christian ('38) that riboflavin is a constituent of the "yellow enzyme," would indicate that this component of the vitamin B complex is concerned with oxidative enzymic processes essential for normal metabolism. A deficiency of riboflavin may then result in a waste of products of metabolism with resultant final collapse of the animal organism.

SUMMARY

This study was carried out with ten pairs of male rats by the paired feeding method of experimentation on two types of diets. The average gain in body weight of the riboflavin deficient animals, during an average experimental period of 125 days, was 6.1 gm. per rat, while during the same period of time on the same amounts of the same ration the litter mate controls, by virtue of having received 20 μg. riboflavin daily, gained 61.3 gm. per animal. To demonstrate the greatest influence that riboflavin exerts in economy of food utilization, experiments should be started on animals weighing 55 to 70 gm. rather than 30 to 45 gm. Experimental evidence has been submitted showing that, unlike in thiamine deficiency which is accompanied with a progressive inanition ultimately approaching starvation, in riboflavin deficiency there is no complicating marked anorexia in the terminal stages of the disease. There may be a moderate reduction, no reduction, or even an increased food intake in the last stages of riboflavin avitaminosis.

The riboflavin deficient rats show alopecia, dermatitis at the denuded areas of the skin, rough hair, conjunctivitis, keratitis, and premature senility.

Since riboflavin is concerned with oxidative-enzymic processes essential for normal metabolism, it is suggested that a deficiency of this vitamin may produce a waste of products of metabolism which ultimately results in collapse of the animal organism.

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THE PRODUCTION OF HIGH VITAMIN A MILK BY DIET [1]

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It has long been recognized that the carotene content of milk shows a marked seasonal variation coincident with the periods when green feeds are available. Baumann and Steenbock ('33) demonstrated that the carotene content of summer butter was increased as much as 400% over that of winter butter. These investigators found that the increase in carotene was accompanied by a concomitant but less marked rise in the content of vitamin A, the rise in the latter case being only approximately 200%.

Because of the relative difficulty in absorption and metabolism of beta carotene, it has been impossible to increase the carotene or vitamin A in the milk further even by the administration of large quantities of carotene (Baumann, Steenbock, Beeson and Rupel, '34). The attempt to increase the vitamin A content of milk by the administration of supplements of cod liver oil has also proved impractical because of the toxic symptoms produced when large quantities of cod liver oil are fed. The most pronounced effect first described by Golding et al. ('26), and confirmed by others (Petersen, '32; Hart, Hadley and Humphrey, '32; McCay and Maynard, '35; McCay, Paul and Maynard, '38), is the prompt lowering in the level of

[1] This work was assisted by a research grant from California Packing Corporation. Dr. J. P. Nutall and Dr. C. M. Bonynge of the Los Angeles Certified Milk Commission assisted in the planning of the experiments. The tests on the cows were made with the cooperation of the Adohr Milk Farms, Los Angeles

butterfat in the milk. Golding ('28) reported that the non-saponifiable fraction of the cod liver oil fails to produce this effect, a result corroborated by McCay and Maynard ('35). The latter investigators found that the triglyceride portion is responsible for the decrease in milk fat while shark liver oil and salmon oil were found to be free from this property. After hydrogenation the toxic effect of cod liver oil is largely lost (McCay, Paul and Maynard, '38). Golding and Zilva ('28) reported that more than 56 gm. of cod liver oil per cow was necessary to produce this effect while the doses employed by McCay and Maynard were considerably higher ($\frac{1}{3}$ cc. per kilo). Hilditch and Thompson ('36) found that the administration of cod liver oil increased the secretion of the unsaturated fatty acid of the C 20-22 series. However, salmon oil which is also rich in these acids fails to cause the decrease in milk fat with the constancy of cod liver oil.

The recent availability on the market in large amounts of shark liver oil which is far richer in vitamin A than cod liver oil and which contains comparatively small amounts of vitamin D has prompted the study of its effect on the nutrition of cows. The present report gives the results of such administration on the composition of the milk in vitamin A while another paper (Deuel, '41) describes the effect of this supplement on the milk and butterfat production.

EXPERIMENTAL

The experiments were carried out on Guernsey cows of a large certified dairy. The animals received a diet which consisted of approximately 5 pounds of a mixture composed of barley, bran, cottonseed press meal, copra, grape residue, and yeast (70 gm.) which was fed twice daily, and of alfalfa fed ad libitum. During all except the last part of the test, when baled alfalfa was used (after December 1st), the alfalfa was fresh-cut. However, since both the control cows (which received only this basal diet) and the experimental cows (which were fed also the vitamin A supplement) were given the

same food, the differences in the vitamin A secretion must be ascribed to the supplement.

The first series of tests were carried out on twelve cows from August 5, to December 23, 1940, although several of the experiments were continued until February, 1941. After a preliminary basal period of 5 weeks during which weekly determinations were made of the carotene and vitamin A content of the milk, shark liver oil [2] was administered in two equally divided doses of 30 cc. daily (approximately 700,000 I.U.) to six of the cows (supplement groups I and II) while the remaining animals (control group) continued on the basal diet without supplement throughout the experiment. Milk samples were collected from the control and supplement groups at 2, 3 and 9 weeks during this period. At the beginning of the eleventh week during the experimental period, the vitamin A supplement was doubled in the case of three cows (supplement group II) to 60 cc. daily containing approximately 1,400,000 I.U. of vitamin A. The remaining three cows in supplement group I continued on the lower dose. Samples were obtained during the fifteenth week of the experiment as well as during the nineteenth and twenty-third weeks in several cases.

A second series of tests (supplement group III) was made on six cows from November 11, 1940, to January 27, 1941. After a preliminary basal period of 1 week, these cows were given 10 cc. of shark liver oil daily (233,000 I.U.) for 6 weeks after which the supplement was doubled to 20 cc. (467,000 I.U.). Samples were collected during the second and fifth weeks on the lower dose and the fourth week after initiation of the higher dosage.

Records of the weight of the milk produced were maintained throughout these experiments. When the samples were collected periodically for the analysis of carotene, vitamin A, and butterfat, approximately 50 cc. aliquots were saved from

[2] Shark liver oil having a vitamin A potency of 25,000 U.S.P. units per gram was furnished by California Packing Corporation in the form of its Dairy Grade "Anim-A" Brand Oil.

each milking and kept in the refrigerator until the end of the week; the fourteen aliquots were mixed, a new aliquot taken for fat analysis and the balance churned in a small motor-driven Dazey churn after souring overnight with a culture of lactic acid bacilli. The induced souring of the milk prior to churning did not result in any demonstrable effect upon the carotene or vitamin A content of the milk. After collecting the butter by pouring through cheesecloth, it was washed to remove the casein, melted in the incubator and allowed to settle out after which it was carefully decanted through anhydrous Na_2SO_4 on a filter to remove any water in the fat layer.

Carotene was determined by the method of Koehn and Sherman ('40) making use of the Leitz-Mass colorimeter employing a filter with maximum transmission at 440 m.μ in place of the Evelyn colorimeter used by the other investigators. Although a preliminary removal of the xanthophyll by extraction with 85, 90 and 92% methyl alcohol was not employed in the individual analyses, the amount of this pigment was found to be appreciable; the corrections found in the determinations made later on composite samples were used for recalculation of the earlier values since it has been shown that xanthophyll is not capable of transformation to vitamin A (Kline, Schultze and Hart, '32).

Vitamin A was determined on the unsaponifiable fraction making use of a Bills-Wallenmeyer electronic photometer which had previously been calibrated with the unsaponifiable fraction of reference cod liver oil. Corrections were made for the absorption due to carotene.

RESULTS

Typical experiments for the first series of tests are given in table 1 while table 2 summarizes the average results obtained in this group of experiments. Table 3 records the data on the fat content of the milk while table 4 gives the results of the second series of tests.

DISCUSSION

When a sufficiently large excess of vitamin A is administered to cows, an increase in the vitamin A content of their milk results. Although the feeding of 10 or even 20 cc. of shark liver oil containing 233,000 and 467,000 I.U. of vitamin A respectively to cows daily did not result in an increased level of vitamin A in the milk, a uniform and marked rise followed the dose of 30 cc. containing 700,000 I.U. A more pronounced increase in the quantity of vitamin A eliminated resulted when the threshold level was further exceeded by doubling the dosage to 60 cc. of shark liver oil containing

TABLE 1

The beta carotene and vitamin A per gram of butterfat in the milk of two Guernsey cows during a basal period followed by an experimental period when the basal diet was continued (control cow 5) or when it was supplemented with shark liver oil (cow 10).

SAMPLE FROM WEEK STARTING	BETA CAROTENE IN MICROGRAMS		VITAMIN A IN I.U.[1]		TOTAL VITAMIN A IN I.U.	
	Cow 5	Cow 10	Cow 5	Cow 10	Cow 5	Cow 10
Basal period						
1940						
8–5	10.8	8.9	25	24	34	32
8–12	7.7	11.5	49	19	49	29
8–19	9.1	9.8	29	20	37	29
8–26	12.3	7.1	47	34	57	40
Average	10.0	9.3	38	24	44	32
Experimental period[2]						
9–16	14.6	10.8	37	67	49	76
9–23	16.2	10.8	19	66	32	75
11–4	13.3	7.4	21	63	32	69
12–16	12.5	4.6	..	123	..	127
1–12–41[3]	5.7	2.5	26	170	31	172
2–12–41[3]	....	3.0	..	168	..	170

[1] International Units.

[2] 700,000 I.U. of vitamin A administered to cow 10 daily after September 9th and 1,400,000 units daily after November 18th. No vitamin A supplement was given to control cow 5.

[3] One-day sample only.

approximately 1,400,000 I.U. daily. After 3 weeks on this higher level, the average vitamin A content of the butterfat had increased to 113 I.U. per gram while after 7 and 11 weeks values of 170 I.U. were noted with one animal. It would seem probable that such an increase might become greater with a still higher intake of vitamin A.

Although the vitamin A level is based largely on spectrophotometric determinations, a bioassay carried out on the butterfat of a control cow (no. 5) and an oil-fed cow (no. 10)

TABLE 2

Summary table showing average beta carotene and vitamin A per gram of butterfat in the milk of Guernsey cows during a basal period followed by an experimental period when the basal diet was continued (control cows) or when it was supplemented with shark liver oil (supplement group). Six cows in each group.

SAMPLE FROM WEEK STARTING	BETA CAROTENE IN MICROGRAMS		VITAMIN A IN I.U.[1]		TOTAL VITAMIN A IN I.U.	
	Control cows	Supplement-fed cows	Control cows	Supplement-fed cows	Control cows	Supplement-fed cows
Basal period						
1940						
8–5	10.8	9.9	34	34	43	42
8–12[2]	12.5	12.0	33	24	43	34
8–19	12.2	13.1	25	24	36	33
8–26	10.9	9.9	41	39	50	48
Average	11.6	11.2	33	40	43	39
Experimental period[3]						
9–16	13.4	12.4	30	59	41	69
9–23	14.2	12.0	23	52	35	62
11–4	14.4	8.3	24	57	36	64
12–16[4]	13.2	7.5(I)	44	65	55	72
12–16		6.8(II)		107		113

[1] International Units.

[2] Five experiments only.

[3] 700,000 I.U. of vitamin A administered to all supplement-fed cows daily starting September 9th to November 18th. Supplement group I consisting of three cows continued at this level until end of experiment and dose to supplement group II consisting of three cows was increased to 1,400,000 I.U. of vitamin A on November 18th.

[4] Four experiments only in control group.

TABLE 3

The fat content of milk in control cows receiving a basal ration throughout and in cows receiving a vitamin A supplement after a previous basal period.

DIET	COW NO.	BUTTERFAT IN PER CENT								
		BASAL PERIOD					EXPERIMENTAL PERIOD			
		8–5	8–12	8–19	8–26	Avg.	9–16	9–23	11–4	12–16
Control group	2	4.8	5.0	5.0	5.1	5.0	5.3	5.4	5.9	6.5
	4	5.0	4.9	5.0	5.3	5.0	5.3	5.6	6.0	..
	5	4.7	4.6	4.8	5.0	4.8	4.4	5.1	5.4	5.5
	9	4.3	4.3	4.4	4.7	4.4	4.6	4.4	5.3	5.7
	11	3.5	3.7	3.7	3.7	3.6	3.9	4.1	4.1	4.4
	12	4.1	4.2	4.1	4.2	4.2	4.2	4.6	4.9	5.4
Average		4.4	4.4	4.5	4.7	4.5	4.6	4.9	5.3	5.5
Supplement group I	1	4.2	4.0	4.1	4.2	4.1	4.6	4.6	5.1	5.3
	6	3.9	4.1	4.0	4.3	4.1	4.4	4.8	4.7	6.0
	8	3.8	3.9	4.3	4.0	4.0	4.2	4.4	4.6	4.8
Supplement group II	3	4.3	4.4	4.4	4.5	4.4	4.5	4.6	5.2	5.5
	7	4.0	4.0	4.0	4.2	4.0	4.4	4.8	4.7	5.4
	10	4.2	4.2	4.4	4.7	4.4	4.5	4.6	4.9	5.4
Average		4.1	4.1	4.2	4.3	4.2	4.4	4.6	4.9	5.4

TABLE 4

The beta carotene and vitamin A in the butter of Guernsey cows during a basal period followed by a period when this diet was supplemented with vitamin A.

COW NO.	CAROTENE IN GAMMA PER GRAM [1]				VITAMIN A IN I.U.[2] PER GRAM				TOTAL VITAMIN A PER GRAM			
	Basal period 11–11 1940	Period 1 (10 cc.)		Period 2 (20 cc.)	Basal period 11–11 1940	Period 1		Period 2	Basal period 11–11 1940	Period 1		Period 2
		11–25 1940	12–16 1940	1–20 1941		11–25 1940	12–16 1940	1–20 1941		11–25 1940	12–16 1940	1–20 1941
13	14.4	10.0	8.3	5.8	24	33	..	33	36	42	..	38
14	17.4	12.9	8.0	6.4	34	28	30	22	49	40	37	27
15	14.3	10.7	7.4	5.2	13	29	32	37	25	38	39	41
16	14.8	12.6	6.1	6.4	29	40	34	35	42	52	39	40
17	17.7	9.8	9.1	3.1	30	34	26	29	45	42	34	32
18	13.4	14.6	9.6	5.8	34	39	..	40	45	51	..	45
Avg.	15.3	11.8	8.1	5.4	27	34	30	33	40	44	37	37
Control Avg.	14.4	....	13.2	...	24	..	44	..	36	..	55	..

[1] Based on correction factor of 0.75.

[2] International Units.

indicated that the differences obtained by the physical methods were approximately correct. The bioassay for butter 5 (1-12-41) was found to be 21.2 I.U. per gram compared with a value of 31 obtained by chemical examination while the value for butter 10 (1-12-41) exceeded 155 I.U. per gram compared with 172 found on the photometer.

The increases which were noted were consistent in each case where 30 cc. of the shark liver oil or more was fed. It is surprising that it is possible to raise the level of vitamin A in the milk of cows receiving a diet of fresh-cut alfalfa designed to give the maximum levels of vitamin A in the butterfat by natural means. The vitamin A contents of butter of all the cows during the basal period and of the control cows during the experimental period which averaged from 33 to 55 I.U. per gram are in the maximum range reported for butter produced when the cows were on green pasturage.

The carotene is calculated on the basis of 1.2 μg. being equivalent to an International Unit which would account for a 50% utilization. This seems justified on the basis of numerous reports of the comparative effectiveness of carotene and vitamin A on various species. Approximately four times as much carotene as vitamin A was required to supply an A deficiency of rats as judged by vaginal smear method (Goss and Guilbert, '39) while three times as much carotene was necessary to prevent night blindness in cattle (Guilbert, Miller and Hughes, '37). On man the effectiveness has been considered to be represented by a ratio of 1.6 to 1.8 (Booher et al., '39) and 2.0 (Wagner, '40).

Only approximately 3% of the ingested vitamin A is excreted in the milk. In view of the higher levels of vitamin A noted in the later samples of cow 10, it is possible that the efficiency of the excretion may be higher when the tissues become thoroughly saturated with this vitamin. Baumann, Steenbock, Beeson and Rupel ('34) have reported an efficiency of only 1.3% for carotene when fed at high levels.

Although a seasonal decrease in milk carotene was noted, the decrease was greater in the vitamin A-fed cows than the

controls. The decrease resulted also after the administration of the 10 cc. dosage where no increased vitamin A excretion was noted. Whether the transformation of carotene to vitamin A was accelerated in the presence of high concentrations of vitamin A or whether the lower output of this pigment resulted from a decreased intake of the carotene-containing feeds is now being investigated.

No toxic symptoms were noted by the continued ingestion of the shark liver oil for over 3 months at a level of 30 cc. daily to three cows; moreover, three cows received this level for 2 months and 60 cc. for 1 month without ill effects while one cow continued on the higher level for 3 months in apparently good health. The best indication of their nutritional condition is to be obtained from the level of their milk production which averaged approximately 10% higher than that of the controls while the butterfat excretion was increased to a somewhat greater extent (Deuel, '41). There is no evidence of the decline in butterfat in the milk similar to that caused by cod liver oil at considerably higher levels. The milk had no taste of the fish liver oil.

SUMMARY

The vitamin A content of the butterfat obtained from cows on a diet high in fresh alfalfa was considerably increased by the administration of shark liver oil in daily doses of approximately 700,000 I.U. although lower amounts were ineffective.

The vitamin A in butterfat averaged 113 I.U. after administration of the vitamin A supplement at a level of 1,400,000 I.U. daily. In one cow, the level reached 170 I.U. per gram which value was also noted a month later. The increased amounts of vitamin A in the butters persisted without diminution over a 5-month period during which the experiments were continued.

There is a marked decrease in carotene which occurs even when doses of shark liver oil, too small to cause an increase in the level of vitamin A in milk, are fed.

No toxic-symptoms were noted and the cows remained in good nutritional condition as reflected by the increased production of milk and butterfat.

The present experiments emphasize the lack of correlation between color of the milk and its vitamin A content.

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THE EFFECT OF CERTAIN ORGANIC COMPOUNDS AND OTHER DIETARY SUPPLEMENTS ON PEROSIS [1]

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TWO FIGURES

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Previous experiments (Jukes, '40 a, b, '41 a, b) have shown that choline is essential for the prevention of perosis in turkeys and chicks. Hogan and co-workers ('41) and Hegsted et al. ('41) have also reported evidence of the anti-perotic effect of choline on chicks. The present communication describes further experiments dealing with the relation of choline and other dietary supplements to perosis.

EXPERIMENTAL

Single-comb White Leghorn chicks were placed in electrically-heated battery brooders at hatching and were fed the experimental diets immediately. From ten to twelve chicks were used in each group. The principal ingredients in the basal diets were crude glucose; [2] washed casein; a low choline strain of brewers' dried yeast; [3] powdered gelatin; gum arabic, U. S. P. powder; salt mixture (bone ash, 38 parts; $CaCO_3$, 20; iodized salt, 22; $MgSO_4$, 3.9; KH_2PO_4, 7.5; ferric citrate, 3.5; $MnSO_4$, 2; copper carbonate, 0.1; ZnO, 0.1); crude soybean oil; and fish oil blend, 3000-A, 400-D.[4] A test period of 28 days

[1] Presented at the annual meeting, American Institute of Nutrition, Chicago, April, 1941.

[2] "Cerelose."

[3] Anheuser-Busch, Strain G.

[4] "Sardilene 400."

was usually employed. Examination for perosis was made as previously described (Jukes, '40 a, b).

Investigations by Stokstad, Almquist and co-workers ('39, '40, '41) have led to the identification of a growth factor for chicks termed the "rice factor." The rice factor was shown by these workers to consist of two components: a protein component supplying glycine and arginine and replaceable by creatine, and a carbohydrate component which could be supplied by adding gum arabic, sodium alginate, glucuronic acid, gluconic acid, galactonic lactone, arabinose or xylose to the

TABLE 1

Effects of choline and the two components of the rice factor upon growth and perosis in chicks on a basal diet deficient in these three nutritional essentials.

Basal diet: Glucose, 53; casein, 18; yeast, 6; salt mixture, 5; soybean oil, 5; fish oil blend, 0.03.

ADDITION TO 87 GM. BASAL DIET	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	24 days	28 days	
	%	%	gm.
8 gm. casein + 5 gm. glucose	0	0	71
8 gm. casein + 5 gm. gum arabic	0	0	80
8 gm. gelatin + 5 gm. glucose	37	100	66
8 gm. gelatin + 5 gm. gum arabic	62	62	90
8 gm. casein + 5 gm. glucose + 0.1 gm. choline	0	0	89
8 gm. casein + 5 gm. gum arabic + 0.1 gm. choline	0	0	75
8 gm. gelatin + 5 gm. glucose + 0.1 gm. choline	0	0	135
8 gm. gelatin + 5 gm. gum arabic + 0.1 gm. choline	0	0	162

diet. It was previously noted that turkeys (Jukes, '40 a, b) but not chicks (Jukes, '41 a) developed perosis on a diet deficient in the rice factor, and an experiment was devised to investigate the relation of choline and the two respective components of the rice factor supplied by gelatin and gum arabic to growth and perosis. The results of this experiment are shown in table 1. Perosis was not produced and the growth-promoting effect of choline was not exerted or was greatly diminished when gelatin was absent from the diet. If gelatin was added and choline omitted, the growth-promoting action of gelatin was absent. However, gum arabic was able to promote growth even in the absence of choline. Gum arabic was found to be

necessary for maximum growth, confirming the findings of Almquist, Stokstad and co-workers.

In experiment 2, gum arabic was included in the diet, and the effect of creatine as a source of the protein component of the rice factor was studied. The results are shown in table 2. Creatine, like gelatin, provoked perosis in the absence of choline. Figure 1 illustrates the tibiae of representative birds in this experiment.

In succeeding experiments the rice factor was included in the diet, which had the following composition: Glucose,[5] 53; washed casein, 18; gelatin, 8; yeast,[6] 6; gum arabic, 5; salt

TABLE 2

Effects of choline and creatine on growth and perosis

Basal diet: Glucose, 53; casein, 26; yeast, 6; salt mixture, 5; gum arabic, 5; soybean oil, 5; fish oil blend, 0.3.

ADDITION TO 100 GM. BASAL DIET	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	21 days	28 days	
	%	%	gm.
None	14	14	83
0.1 gm. choline	0	0	82
1.0 gm. creatine	50	75	106
0.1 gm. choline + 1.0 gm. creatine	0	0	180

mixture, 5; soybean oil, 5; fish oil blend,[7] 0.3 (diet 115). This diet was used in subsequent experiments on choline deficiency in chicks. On this diet it was found that chicks usually averaged between 100 and 120 gm. in weight at 28 days with an incidence of perosis of 80% to 100%. Usually the growth rate was approximately doubled and perosis was prevented completely by adding 0.1% of choline to the diet. However, this level of choline is marginal for the prevention of perosis under such conditions, and occasionally a mild case appeared. The growth-promoting effect of choline was evident to a significant degree when choline was added at levels as low as 0.01% which had no discernible effect on perosis.

[5] See footnote 2.
[6] See footnote 3.
[7] See footnote 4.

The effect of a secondary deficiency in preventing the appearance of perosis was also noted under a different set of conditions. In this case, gelatin was included in the basal diet but casein was omitted, and a sample of meat scrap was used as the source of protein. Simultaneously, part of the

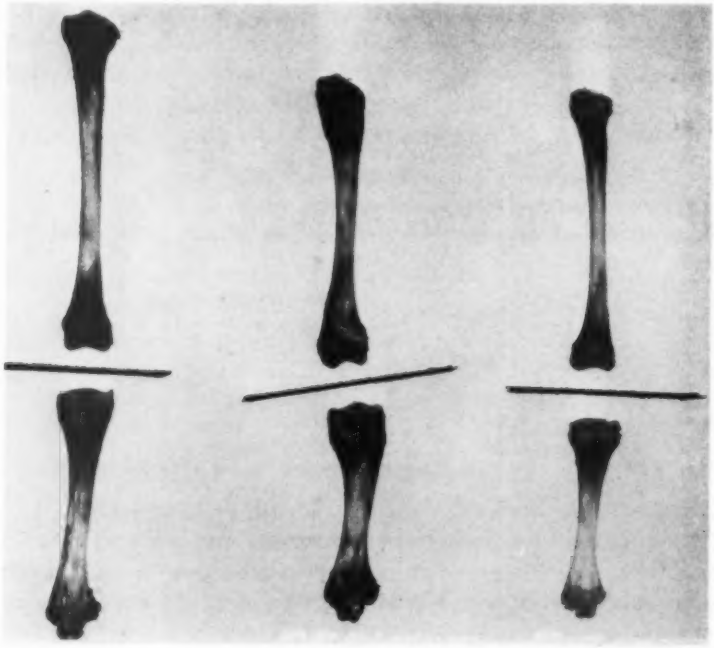

Fig. 1 Tibiae and metatarsi of chicks in experiment 2 at 28 days of age on (a) basal diet + 1% creatine + 0.1% choline, (b) basal diet + 1% creatine, (c) basal diet. The lines have been added to indicate the direction of the transverse plane of the hock joints.

minerals were omitted because of the high mineral content of the meat scrap. Growth was very slow and was not increased by the addition of choline. Perosis did not appear. However, when casein was added and choline omitted, perosis was produced. The experimental data are summarized in table 3.

Evidently casein "provoked" perosis in a manner similar to gelatin, although gelatin was present in the basal diet.

Experiments with manganese deficiency. If manganese was omitted from the salt mixture, diet 115 contained only 2.3 p.p.m. of manganese, which is not sufficient to protect against perosis (Gallup and Norris, '39). An experiment was made with this modification of the basal diet and the results are

TABLE 3

Aggravating effect on perosis of casein when added to a diet in which meat scrap was the principal protein source.

SERIES NO.	DIET NO.	CHANGES IN DIET 115	INCIDENCE OF PEROSIS AT 28 DAYS	GAIN IN 28 DAYS
			%	gm.
1	1	None	71	48
1	2	22 gm. meat scrap + 0.5 gm. NaCl + 0.05 gm. $MnSO_4$ replacing 18 gm. casein + 5 gm. salt mixture	0	37
1	3	Diet no. 2 + 0.1% choline	0	38
1	4	Diet no. 1 + 0.1% choline	0	152
2	1	None	89	76
2	5	Diet no. 2 + 8% casein	56	118
2	4	Diet no. 1 + 0.1% choline	0	150

illustrated in figure 2. Perosis developed and growth was slow in the absence of either manganese or choline. The appearance of the perosis in the manganese-deficient birds was similar to that in the choline-deficient birds. This confirms the observations of Hogan, Richardson, Patrick and Kempster ('41) who found that manganese and an organic factor were both necessary for the prevention of perosis.

It was suggested (Jukes, '41 a) that the tension exerted on the bones by the muscles may play a part in causing the distortion of the bones which characterizes perosis and if the tension is reduced by muscular dystrophy as in creatine deficiency, the tendency toward perosis may be lessened. This suggestion was made as a result of the observation that perosis did not develop on a diet which was deficient both in creatine or one or more of its precursors and in choline. As

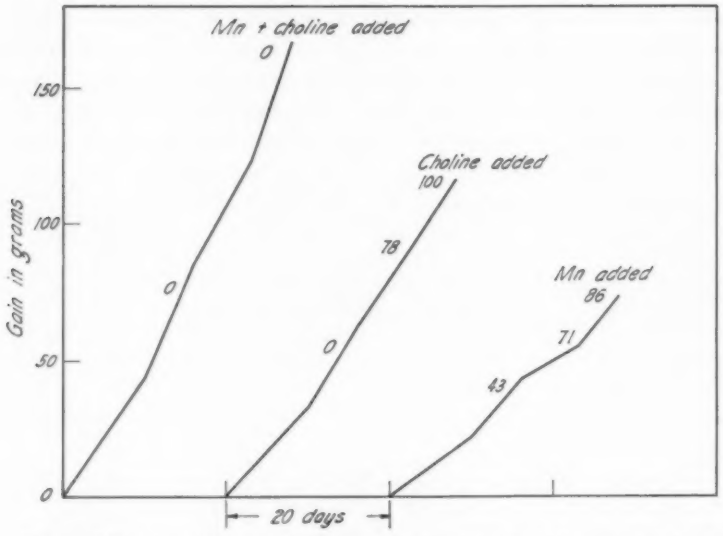

Fig. 2 Effects on chicks of additions of manganese and choline, separately and in combination, to a diet deficient in both these substances. The figures above the curves indicate the per cent incidence of perosis.

a further test of the suggestion, an experiment was made with a diet from which manganese and gelatin were omitted. The results are shown in table 4. Creatine deficiency reduced somewhat the severity of the perosis caused by a lack of manganese but the effect was not so clear-cut as that obtained

TABLE 4

Effects upon growth and perosis in chicks produced by adding choline but omitting manganese and gelatin from diet 115.

Basal diet: Glucose, 53; casein, 18; yeast, 6; gum arabic, 5; salt-mixture (Mn omitted), 5; soybean oil, 5; fish oil blend, 0.3; choline, 0.1.

ADDITION TO 92 GM. BASAL DIET	INCIDENCE OF PEROSIS AT			GAIN IN 28 DAYS
	15 days	20 days	28 days	
	%	%	%	gm.
8 gm. casein	14	43	43	89
8 gm. gelatin	33	78	100	116
8 gm. casein + 0.1 gm. $MnSO_4$	0	0	0	111
8 gm. gelatin + 0.1 gm. $MnSO_4$	0	0	0	166

in the case of perosis caused by a lack of choline (Jukes, '41 a, also vide supra).

Effect of methionine. Methionine was shown by Tucker and Eckstein ('37) to have a lipotropic action similar to that of choline (Best and Huntsman, '32). Griffith and Mulford ('41) found that methionine could replace choline in the prevention of hemorrhagic kidneys in young rats, 1 part of choline being replaceable by 3 to 4 parts of methionine. A direct explanation of these phenomena is probably furnished by the observation that methionine may act as a precursor of choline in vivo in the case of rats (du Vigneaud, Chandler, Cohn and Brown, '40). However, methionine at a level of 0.2% was found to be ineffective as a substitute for choline in the

TABLE 5

Effects of additions of methionine, methionine plus aminoethanol, and choline to diet 115.

ADDITION TO 100 GM. DIET 115	INCIDENCE OF PEROSIS AT		GAIN IN 24 DAYS
	15 days	24 days	
	%	%	*gm.*
None	80	89	64
0.5 gm. methionine	67	75	62
0.5 gm. methionine + 0.3 gm. aminoethanol	83	89	67
0.1 gm. choline	0	0	107

prevention of perosis in turkeys (Jukes, '41 b). In view of the results of Griffith and Mulford, it seemed desirable to perform another experiment with methionine and to feed it to chicks at a higher level than was used previously. Aminoethanol was also added to the diet of one group with the thought that methionine might furnish the methyl groups (du Vigneaud, Chandler, Cohn and Brown, '40) and aminoethanol the remaining moiety of the choline molecule. The results are shown in table 5. Methionine was completely ineffective as a precursor of choline, which is not surprising in view of the fact that acute choline deficiency in chicks is produced by diet 115 which contains 18% of casein. Casein contains about 3% of methionine (Baernstein, '36).

Extraction of the anti-perotic factor from natural foods. Choline exists in nature largely in the form of phospholipins such as lecithin which is to some extent in loose combination with proteins. In such combination, lecithin is not extractable with fat solvents until the lecithoproteins have been subjected to vigorous treatment, such as boiling with alcohol. The action of boiling alcohol on soybean meal was investigated as follows: 1 kilo of commercial soybean meal was boiled for 1 hour with 4 liters of 95% ethanol. The mixture was filtered, and the residue was washed with hot ethanol. The entire process was repeated, and the residue was washed with hot ethanol. The residual soybean meal was dried at room temperature. The

TABLE 6

Effect of extraction with boiling alcohol upon the anti-perotic activity of soybean meal.

SUPPLEMENT TO DIET 115	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	21 days	28 days	
	%	%	*gm.*
None	75	100	74
Residue from 30% soybean meal[1]	70	90	141
30% original soybean meal[1]	0	0	202
0.1% choline	0	0	142

[1] Replacing 12 parts of casein and 18 parts of cerelose in diet 115.

combined alcoholic extracts were evaporated to small volume under reduced pressure. The residue was compared with the original soybean meal in a feeding experiment which is summarized in table 6. Treatment with alcohol evidently removed the anti-perotic factor from soybean meal, but the residue still supplied a growth-promoting fraction which was absent from the basal diet. The alcohol-soluble material was fed in a separate experiment and was found to promote growth and protect against perosis almost completely when fed at a level corresponding to 40% of soybean meal. An alcoholic extract was similarly prepared from meat scrap and was found to be growth-promoting and partially protective against perosis. When fed at a level corresponding to 30% meat scrap,

the effect was similar to that obtained from about 0.07% choline. These results do not show that the anti-perotic factor in soybean meal and meat scrap is identical with choline, but the experiments indicate that the factor has certain properties corresponding to those of choline.

DISCUSSION

It was observed (Jukes, '39) that manganese under certain dietary conditions accelerated the appearance of perosis in turkey poults. Later it was found that the basal diet used was deficient in choline, and thus it became evident that high levels of manganese had no "sparing action" on choline.

The complete and surprising inability of methionine to replace choline in the case of the chick serves to contrast this species with the rat. The anti-perotic activity of foods, hence, cannot be related to their methionine content. This statement is further supported by the observation that the anti-perotic activity of soybean meal and meat scrap was removed by boiling alcohol which would not extract methionine from the proteins of these materials.

In this investigation, as previously (Jukes, '41 a), the feathers of birds on diets deficient in creatine or its precursors were "frayed" (Warren, '38) in appearance due at least partly to imperfect development of the barbules and sparseness of the barbs. The same appearance was noted to a marked degree in birds on diets 2 and 3 in table 3, which contained no casein.

Perosis has also been observed (McElroy and Jukes, '40) as a symptom accompanying the egg white syndrome in chicks. The basal diet contained manganese, and in one experiment 0.5% of choline was added to the basal diet without any noticeable effect in preventing perosis. The perotic symptoms were characterized by a marked distortion of the shafts of the tarsi, recalling the symptoms described by Buckner et al. ('32), rather than by the looseness and dislocation of the tibio-tarsal joints noted in the cases of deficiencies of manganese or choline.

Several nutritional conditions are now known to affect perosis. Perosis is aggravated by dietary creatine and its precursors in gelatin and may also be aggravated under certain conditions by casein. Perosis may be produced or aggravated by the addition of raw egg white to the diet, and by the addition of excessive quantities of mineral supplements such as bone meal (Card, '29), magnesium carbonate (Buckner et al., '32), and ferric citrate (Wilgus and Patton, '39). Perosis is prevented by manganese (Wilgus, Norris and Heuser, '36), by choline either free or in the form of lecithin (Jukes, '41 b), by certain choline analogues (Jukes, '40 b; Jukes and Welch, unpublished), and possibly by certain unidentified factors.

SUMMARY

1. A diet is described for the production of acute choline deficiency in chicks. The deficiency is characterized by slow growth and perosis.

2. Perosis did not develop if creatine or gelatin was omitted from the basal diet, nor did perosis develop if the casein in the basal diet was substituted by meat scrap, but if casein and meat scrap were both included perosis developed.

3. Manganese and choline were both found to be necessary for growth and for the prevention of perosis in chicks.

4. Methionine at a level of 0.5% was found to be without effect on choline deficiency in chicks, although 0.1% choline was sufficient to prevent perosis and promote growth when added to the basal diet. This observation contrasts chicks with rats, which are protected from choline deficiency by methionine.

5. The anti-perotic activity of certain natural foods was measured by feeding them in combination with the basal choline-deficient diet. The activity was removed from soybean meal and meat scrap by extraction with boiling alcohol.

6. The nutritional factors affecting perosis are enumerated.

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INFLUENCE OF PROTEIN INTAKE UPON GROWTH, REPRODUCTION, AND LONGEVITY STUDIED AT DIFFERENT CALCIUM LEVELS[1]

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The present paper completes a series of three reports upon our investigation of the effects of simultaneous increases of the protein and calcium contents of a diet already adequate, but suboptimal in calcium, and perhaps also in its protein and riboflavin contents and vitamin A value. Increasing the original 14.4% of protein to 18.8 or to 25% by addition of casein increased the rate of growth and almost correspondingly the amount of calcium in the body at a given age, but the percentage of body calcium was not thereby increased (Conner and Sherman, '36; Conner, Kao and Sherman, '41).

The effects of these increments of protein intake upon the nutritional well-being of animals throughout their life cycles have now been studied at two liberal levels of calcium intake: 0.64 and 0.80% of calcium in the air-dry food mixture. The basal dietary of this investigation is more or less closely analogous to widely prevalent conditions of human food supply. The outcome of these experiments should, therefore, have a significant bearing upon the general problem as to what degrees of simultaneous enrichment of the dietary in calcium and protein are most conducive to permanent nutritional well-being.

Our problem is thus quite different from that of Slonaker ('38, '39), who studied the effects of increasing the protein

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TABLE 1

Records of rats on diets of different protein and calcium contents.

	DIET 170		DIET 171		DIET 673		DIET 172		DIET 675	
Protein in diet	18.8%		18.8%		25.0%		18.8%		25.0%	
Calcium in diet	0.19%		0.61%		0.61%		0.77%		0.77%	
	No. of cases		No. of cases		No. of cases		No. of cases		No. of cases	
Weight at 30 days										
Males, *gm.*	35	52 ± 0.9	52	55 ± 0.5	63	59 ± 0.5	45	51 ± 0.7	51	56 ± 0.6
Females, "	40	50 ± 0.7	70	51 ± 0.5	68	55 ± 0.5	58	48 ± 0.6	59	54 ± 0.6
Weight at 60 days										
Males, *gm.*	30	137 ± 2	47	153 ± 2	51	169 ± 2	41	150 ± 2	39	157 ± 2
Females, "	34	112 ± 1	65	118 ± 1	56	131 ± 1	53	121 ± 1	47	128 ± 2
Weight at 90 days										
Males, *gm.*	24	200 ± 3	42	226 ± 2	39	239 ± 3	36	230 ± 2	29	224 ± 4
Females, "	28	155 ± 3	60	168 ± 3	44	175 ± 2	47	174 ± 2	31	170 ± 2
Weight at 180 days										
Males, *gm.*	15	306 ± 4	33	323 ± 3	27	312 ± 4	27	324 ± 4	15	291 ± 6
Females, "	16	207 ± 2	46	214 ± 2	32	214 ± 3	38	213 ± 2	19	203 ± 3
Weight at 365 days										
Males, *gm.*	7	349 ± 5	28	355 ± 5	13	367 ± 6	22	364 ± 4	4	361 ± 21
Females, "	9	252 ± 3	39	253 ± 2	20	254 ± 4	33	254 ± 2	4	254 ± 5
Age of females at birth of first young, *days*	11	124 ± 5.4	40	102 ± 1.4	18	101 ± 1.9	32	104 ± 2.1	6	100 ± 3.7
Duration of reproductive life, *days*	11	267 ± 30	41	319 ± 9	21	328 ± 24	33	378 ± 13	6	302 ± 37
Young born per female	11	36 ± 4.3	41	43 ± 2.2	21	41 ± 3.3	33	48 ± 1.7	6	45 ± 6.2
Young reared per female	11	23 ± 2.6	41	29 ± 1.7	21	28 ± 2.4	33	32 ± 1.7	6	34 ± 5.8
Average weight of young at 28 days, *gm.*	255	44.5 ± 0.2	1185	47.5 ± 0.1	578	48.8 ± 0.1	1051	46.9 ± 0.1	205	48.4 ± 0.2
Length of life										
Males, *days*	8	674 ± 39	28	714 ± 21	14	684 ± 32	22	720 ± 16	4	699 ± 54
Females, "	11	709 ± 59	40	848 ± 19	21	831 ± 22	33	888 ± 15	6	731 ± 80

content of the diet by addition of lean meat only; and our experiments differ essentially from those of Steenbock, Kent and Gross ('18) in that we have employed not only a different basal diet but also a series of criteria of nutritional well-being which extend to all parts of the normal life cycle. The conditions here are also very different from those of McCay, Maynard, Sperling and Osgood ('41) whose feedings of different levels of protein were begun when the experimental animals were middle-aged.

EXPERIMENTAL

Strictly parallel lots of laboratory-bred rats of the uniform initial age of 4 weeks (three females and two males in each lot) were fed the five diets whose effects are here compared. The protein and calcium contents of each diet are shown in table 1 and the full description may be found, if desired, in the paper of Conner and Sherman ('36, pp. 697 and 704). The original matched lots of experimental animals were continued on their respective diets from the age of 4 weeks (here considered the end of infancy in the rat) until the completion of their natural lives. Many of their offspring were continued on the same family dietaries throughout their entire lives, and in the case of all the diets here discussed except diet 675, typical members of the third generation were also continued on the family dietary. None of the dietaries here studied resulted in any marked change from one generation to another; hence, in our table 1 we give the mean results of the records of all the rats directly compared on the respective diets without distinction between the individuals of the first, second, and third generations.

DISCUSSION

The data shown in table 1, being the results of side-by-side experiments with directly matched initial animals, are strictly comparable. With moderate allowance for experimental variation, these may also be compared with the data for closely related animals which at practically the same time were fed diets 168 and 169 containing 14.4% of protein and, respec-

tively, 0.64 and 0.80% of calcium, as described in another paper from this laboratory (Van Duyne, Lanford, Toepfer and Sherman, '41).

The rate of growth and correspondingly the size at early ages tend to increase with the level of protein fed, but the adult size is essentially the same with 18.8 or 25% of protein, and only slightly greater than with 14.4%.

Similarly the higher protein levels seem to result in very slightly earlier maturity as judged by the age of the females at birth of their first young, but the differences here, particularly between the animals on 18.8 and 25.0% protein, were not much larger than their probable errors and are therefore of doubtful significance.

The higher intakes of protein seem to result in slightly larger offspring, the difference appearing more clearly significant between the animals on diets with 14.4 and 18.8% than between those receiving 18.8 and 25% of protein in the dry food mixture. Thus at the calcium level of 0.61–0.64%, the average weights of the offspring at 28 days were, on the three protein levels, 40.2 $\pm$ 0.1, 47.5 $\pm$ 0.1, and 48.8 $\pm$ 0.1 gm., respectively. A similar relationship holds for the results of the three levels of protein feeding when the calcium content of the dietaries is 0.77–0.81%.

Thus the optimal degree of enrichment with protein seems to have been reached at a level of about 18% of protein in dietaries which also contained the favorable calcium content of about 0.6% of the dry food. These figures seem clearly to be within the range of the optimal plateau.

CONCLUSIONS

Within the range here studied, 14.4 to 25% of protein in the dry food mixture, increased protein content of the diet tended to increased growth up to about 60 days of age in the rat. This was found for both sexes and for both the basal diets used, containing respectively 0.61–0.64 and 0.77–0.81% of calcium in the dry matter.

At 6 months and at 1 year of age the rats which had received diets with 18.8 and 25% of protein respectively, were of essentially the same size, and slightly larger than those which received the diet with 14.4% protein.

With the higher protein intakes, maturity appeared to be reached slightly earlier as judged by the age of the females at birth of first young.

The average numbers of young born, and of young reared, per female, were essentially the same on the diets of 18.8 and 25% protein respectively. The duration of reproductive life was also essentially the same on the different protein levels here studied. The higher levels of protein resulted in slightly higher average weight of young at 28 days of age.

With each sex and with each calcium level, the average length of life was essentially the same on the different protein levels here studied.

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